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SYMPOSIUM ON CROWDING, STRESS, AND NATURAL SELECTION

BY INVITATION OF THE COMMITTEE ON ARRANGEMENTS FOR THE AUTUMN MEETING

Presented before the Academy, November 15, 1960

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CHAIRMAN'S OPENING REMARKS

BY CARLETON S. COON

THE UNIVERSITY MUSEUM, UNIVERSITY OF PENNSYLVANIA

As the population of the world grows at compound interest every year, we need to know what happens to animals, including *Homo sapiens*, when their territories become overpopulated. In this symposium we shall hear reports, based on observation and experiment, on the physiological effects of crowding and stress brought about by overloads of intraspecific interaction among several animal species.

Anthropologists seeking to explain the evolution of man are coming to realize that it was not only the acquisition of tool-making and speech that made our ancestors human, but also their ability to tolerate each other's presence. There can be little doubt that our ancestors were genetically changed by natural selection so that they could live in increasingly larger and more complexly organized social aggregations. Now that the rate of human population increase has itself increased to unparalleled proportions, the problems of food supply and standing room become insignificant and academic compared to the problem of increasing stress and decreasing sanity, which the following papers will elucidate.

But even more important for present purposes than these practical considerations may be the fact that Christian and his associates have opened a new door to the study of evolution, a non-Malthusian kind of natural selection. Unless I am hopelessly naïve, this symposium should mark some kind of a landmark, of use to workers in many branches of science.

*PHENOMENA ASSOCIATED WITH POPULATION DENSITY**

By John J. Christian

PENROSE RESEARCH LABORATORY, ZOOLOGICAL SOCIETY OF PHILADELPHIA, AND DEPARTMENT OF PATHOLOGY, UNIVERSITY OF PENNSYLVANIA

The mammals and their populations which are the topic of this discussion presumably have evolved adequate means of adapting to their environment and possess sufficient physiologic flexibility to meet daily and seasonal changes in their environment. They also must have developed mechanisms of population control which have prevented overutilization and destruction of their environment and therefore their own extinction. The growth of mammalian populations usually ceases short of this point; yet declines in mammalian populations are widely and loosely attributed to shortages of food. Critical studies generally have failed to associate these declines with exhaustion of the food supply other than in highly localized areas. Any dependable, extensively effective system of population control surely must be density dependent, directly or indirectly, and therefore would be integral to the population itself. Some years ago we proposed that population growth and decline were regulated by series of feed-back mechanisms, particularly involving the pituitary-adrenocortical and pituitary-gonadal systems, and that these in turn were activated by socio-psychological factors (intraspecific competition) within the population.[13, 14] Present evidence indicates that interacting behavioral and endocrine mechanisms comprise at least an important part of such a system in the individuals in a population: a system responding to changes in the number of animals in such a way that population growth is self-limiting and self-regulating. This paper will summarize the evidence for such a mechanism, illustrated to a large degree by examples drawn from data so far unpublished, the details of which will be published elsewhere.

Before proceeding to a summary of the evidence, litter size should be mentioned, since selection for physiological mechanisms regulating population growth undoubtedly involved selection for optimum litter size, defined as the mean number of young per litter sufficient to maintain the population with the least cost to the maternal organism. Regulation of reproductive function is an integral part of the adaptive responses which result in regulating population growth because, to a large degree, they regulate reproduction.[60] Normally, life-maintaining functions and reproduction are not mutually exclusive, but become so when the energy requirements for survival exceed the capacity of the animal to meet them and to reproduce. Reproduction is sacrificed, as we shall see.

The Evidence.—The regulation of population growth, as outlined, involves behavioral and physiological mechanisms, the latter importantly including the pituitary-adrenocortical system as well as pituitary-gonadal function. So much has been written on adrenocortical function that it will not be reviewed here, other than to say that it is vitally important in the regulation of normal metabolic processes, and perhaps more particularly in emergencies. Increased adrenocortical function provides one of the important means of insuring survival when confronted with environmental change or markedly increased physiological demands. Notably, it increases its size and function in response to emotional stress, burns, injury, cold, and a number of other potentially harmful stimuli and therefore provides

an extremely useful indication of the degree of stimulation from adverse circumstances to which an animal has been subjected. For this reason much consideration will be given to the adrenal glands in this discussion (as well as to reproductive

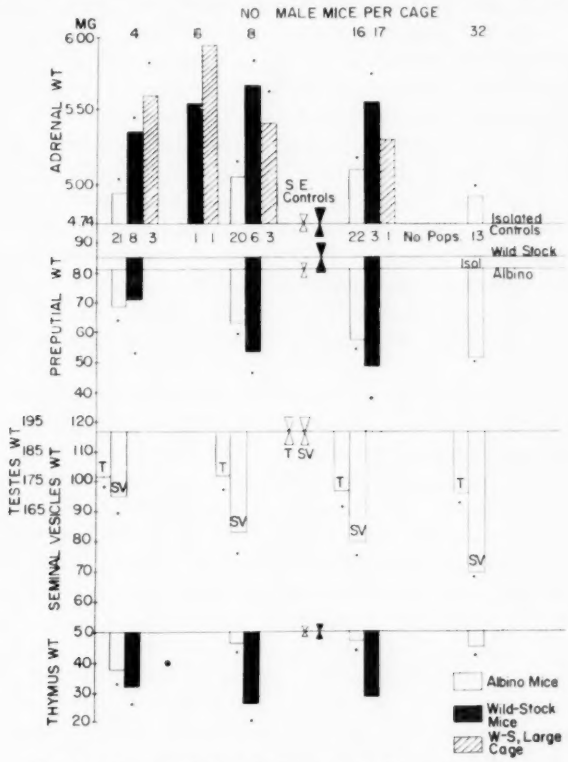

FIG. 1.—Organ weights of albino and wild-stock male house mice from populations of different sizes. Control weights from isolated mice are shown as horizontal lines with their standard errors as triangular areas above and below the line. Standard errors of the experimental mean values are given as dots above the bar for each mean. Experimental values are plotted with respect to their appropriate controls. The control mean weights of the preputial glands differed for the two strains and are plotted separately. Graded responses with increasing size of population is clearly seen, especially for wild-stock mice. The decline in adrenal weight at the largest population for each strain is due to the loss of lipid with a decrease in cellular size although the number of cells continued to increase. Note that the magnitudes of the adrenal responses are similar for wild-stock mice whether the populations were in cages with an area of 1 square foot (solid bars) or 42 square feet (cross-hatched bars), although there appears to be less regularity for the latter, but the irregularity primarily is due to the limited number of populations used.

function) although one must remember that it is only one part of the adaptive mechanism and that the central nervous system, as well as a number of other glands, actively and inseparably participate in physiologic adaptation. The fact

that the adrenal cortex responds to psychological or emotional stresses is a matter of prime importance in the present considerations.

To show that the mechanism outlined above actually is effective in regulating population growth one must demonstrate increased adrenocortical and decreased reproductive functions with increased size of a population. Experiments to explore these relationships were conducted in the laboratory with populations of fixed size, with freely growing populations of house mice and voles and, subsequently, with natural populations of a number of species in the field.

In the first series of experiments, male mice which had been caged singly at weaning were placed in groups of from 3 to 32 per cage (Fig. 1) with adequate numbers kept singly as controls.[14, 20] Food and water were supplied in excess of usage at all times. Organ weights were obtained after sacrifice a week later. The mean organ weight from all of the mice in a population (single cage) was used as the unit of measurement (one degree of freedom) in order to establish that the observed effects are due to changes in the population and not to so-called "within cage" effects,[57, 62] in these and, whenever possible, in all subsequent experiments to be described.

Adrenal weight, presumably a measure of adrenal function under the chronic conditions of these experiments, increased with increasing density of the population in both albino and wild-stock house mice. The increases in adrenal weight were due primarily to cellular hypertrophy and hyperplasia of the *zona fasciculata*. The decline in adrenal weight from maximum values in populations of 17 wild-stock and 32 albino mice resulted from a loss of stored lipid which more than offset the continued increase in cellularity of the *fasciculata*. Thymus weight, using involution of the thymus as an indicator of adrenocortical function, decreased with increased density of population, especially in the wild-stock mice. The weights of the preputial glands, seminal vesicles, and testes declined progressively with increasing density, indicating inhibition of reproductive function at the gonadal as well as at the pituitary level (Fig. 1). The decline in weights of the sex accessories presumably reflects a proportional decline in the secretion of testicular androgens. The lower weight of the preputials in the presence of increased secretion of ACTH (indicated by the increased adrenocortical mass) is especially indicative, as ACTH will stimulate preputial growth in the absence of testosterone.[49] The differences in the degrees of response between the albino and the wild-stock mice is due to the generally greater aggressiveness and reactivity of the latter. It is of particular interest in the present context that we are observing the differences between animals which have been selected to be docile and to be "reproductive machines" over a long period of time and those recently derived from wild stock.

This experimental design was based on the assumption that the stimulus to increased adrenocortical and decreased gonadal function was socio-psychological or competitive in nature, acting through higher centers—the hypothalamus, the anterior pituitary, and thence to the pituitary target glands. Therefore, to check the effects of density *per se* and the possible role of increased exercise, experiments were run with populations of the same size as before, except that the cages had 42 times more area. These results also are shown in Figure 1. In spite of the greater space the adrenal responses were comparable to those in the preceding experiments, although the numbers of populations used were not adequate to determine quanti-

tative relationships with the same degree of precision. Therefore within the spatial limits used the response of the mice was not to density *per se*, but rather to the presence of other mice.

The relationship between physiological response and social factors was further explored in experiments with wild-stock mice in which the responses of individual mice in each of a number of populations were investigated.[35] Mice rank themselves much like chickens, in a series of dominance-subordinance relationships, and this

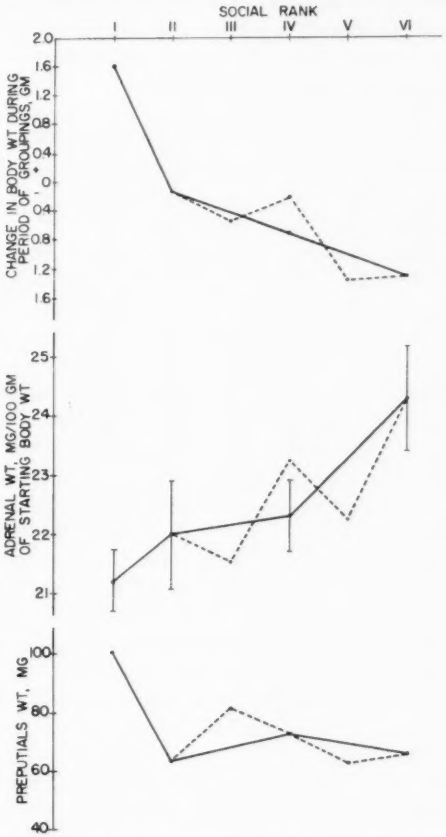

FIG. 2.—Changes in adrenal, body, and preputial weights with social rank for male wild-stock house mice from 14 populations of 6 each. Ranks three, four, and five are combined, as it is difficult to distinguish reliably between them. However, their respective values are indicated by the broken line. Also it is not strictly correct to connect non-parametric points by lines, but then it is done for the sake of clarity. The correct spacing between ranks is unknown and probably is not constant from population to population. The data suggest that the differences between ranks I and II are greater than the differences between other pairs of successive ranks. Only the dominant animals gained weight during the ten day experimental period. Rank II mice maintained their weight, while those in other ranks lost weight.

characteristic was utilized in these experiments.[69] Adrenal weight was least in the dominant animals and greatest in the most subordinate animals, while the mean adrenal weights of animals in the intermediate ranks were strung out in between these two extremes in a more or less linear fashion (Fig. 2). The results from mice in ranks III, IV, and V have been combined in Figure 2, as it is difficult to determine rank accurately in the middle of a hierarchy and such determinations do not have the same reliability as do those for ranks I, II, and VI. Finally, the impression was gained from observing grouped mice that the more clear-cut the rank differences are, the less the physiological response to "density" implying

that populations in which rank differences are clearly established may reach a greater size than those in which rank differences are less well defined. The weights of the preputial glands were reciprocally related to the adrenal weights (Fig. 2), but the weights of other reproductive organs were too variable to support any conclusions. Similar correlations between adrenal weight and dominance-subordinance relationships have been demonstrated for voles[28] and rats.[3] In these and other experiments, inhibition of growth, in addition to an actual loss of weight, has been demonstrated.[15, 70] In the experiments on social rank, only the dominant animals gained weight during the period of grouping, the second ranking animals maintained their status quo, while the remainder lost weight in proportion to descending order of rank (Fig. 2).

Measurements of adrenal steroid secretion have been made in grouped versus isolated guinea pigs,[17] monkeys, and humans,[55] and in each case there was a significantly greater secretion of corticoids by grouped than by isolated individuals. Similarly Eik-Nes[41] found an appreciably higher rate of corticoid secretion in subordinate than in dominant dogs.

These physiologic responses have been attributed to fighting, directly, or to injury resulting from fighting.[28, 65] Experiments to test the effects of injury and fighting show that the adrenal and reproductive responses to grouping occur with or without fighting and irrespective of the absence or, when present, of the degree of injury.[18] However, probably the strongest argument against attributing these effects to fighting is the graded adrenal weight with social rank, with the dominant animal showing little increase in adrenal weight compared to the subordinate animals, whereas the dominant animals fight at least as much and probably more than the subordinate animals.

Competition for food or deprivation of food does not contribute to the observed changes in adrenal weight.[19] In fact, the results of experiments suggest that these animals cannot anticipate a food shortage and, consequently, that each mouse in a population is equally affected by a restricted supply of food, irrespective of its rank. Food, whether abundant or scarce, scattered or localized, did not appear to affect competition and therefore had no effect on adrenal activity in house mice, either directly or indirectly. However, Frank[43] presents evidence that a shortage of food may increase competition in voles (*Microtus arvalis*) and therefore may be effective in other species.

The experiments discussed so far were with males of house mice or other species, but earlier experiments had shown that reproduction by female mice declines with increasing population size, and that the dominant females in a group do most of the reproducing.[30, 58] Subsequently it was shown that maturation was inhibited in grouped, compared with isolated, female mice in the absence of males.[1] In none of these experiments was adrenal function investigated. However, the adrenals of female mice do respond to increased numbers with hypertrophy of the *zona fasciculata* and accelerated involution of the X-zone, and inhibition of uterine size when grouped in the absence of males.[21] The increase in the *zona fasciculata* was significant, but it was far less than that observed in males under similar circumstances and was offset in terms of weight by the involution of the X-zone. The reaction of the females to one another is, alone, inadequate to explain the degree of cortical response of females from populations of mixed sex (cf. below). The

X-zone of the adrenal cortex of the mouse is a transitory zone involuted by androgens and apparently maintained by lutenizing hormone (LH) of the anterior pituitary.[46, 50] Consequently, this zone disappears at puberty in the male, or during first pregnancy in the female.[50, 51] Therefore the involution of the X-zone in grouped female mice suggests an increased secretion of adrenal androgens, a decreased secretion of LH, or both. Additional evidence favors the first explanation.[21]

Increased density affects reproductive performance and lactation of female mice. If females are kept in groups of 20 mice of each sex per cage, for six weeks, and subsequently removed, and then each female placed in a cage with a previously isolated male, the reproductive function of the female is severely curtailed. The growth of the young is also inhibited, as compared to appropriate controls.[26] Many never bear young, although nearly all become pregnant only to resorb the young early in pregnancy. Those that bear litters do so much later than controls placed with males at the same time, and the average number of ovulations and the mean litter size are less. The young nursed by these mice are stunted at the time of weaning (Fig. 3), due to deficient lactation and not to *in utero* effects, as shown by the normal growth of young born to previously crowded mothers which were removed and fostered by previously isolated females (Fig. 3). Finally, these effects on the pups are lasting. When they are maintained as *isolated* pairs of one female and one male each, their offspring are in turn stunted at weaning, although the effect is observable only in litters of 8, 9, or 10 compared to 6 and above for the preceding generation (Fig. 3). When these young are bred, in turn, their pups are stunted, but not significantly. Crowding clearly has a profound effect on every aspect of reproduction, with prolonged effects on the offspring. Similar results have been described from experiments with voles (*Microtus*).[12]

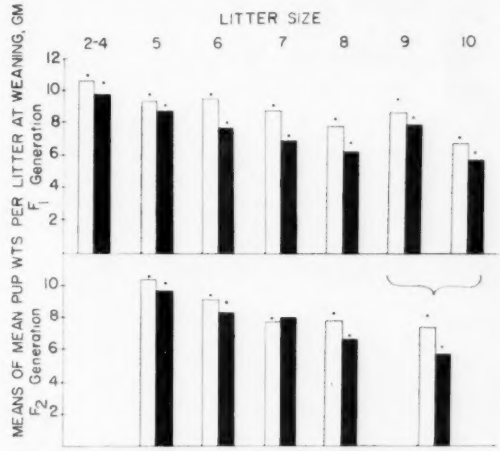

FIG. 3.—Illustrates the degree of inhibition of growth measured at weaning, of mice nursed by previously crowded mothers (solid bars) compared with those nursed by isolated mothers (open bars). There was no difference in the weight of pups at birth whether from previously crowded or isolated mothers. Half litters were exchanged at birth between the two types of mothers so that each mother nursed half of her own and half of those of a mother subjected to the opposing treatment. The young nursed by previously crowded females were in turn placed with a litter-mate of the opposite sex and allowed to breed. The young of these mice in turn weighed less at weaning than those born to and nursed by mothers who, in turn, had been nursed by isolated females. However, the differences were apparent only in litters of 8, 9, or 10 each in this F_2 generation, whereas the difference was clear in litters of 6 or greater in the F_1 generation. Note that in both experimental and control series the mean weights of pups at weaning declined with increasing litter size.

One of the actions of the adrenal glucocorticoids is to decrease resistance to infection by inhibiting inflammation, granulation, phagocytosis, and antibody forma-

tion. These actions have been observed frequently as a result of injecting cortisone, hydrocortisone, or ACTH into animals and humans.[59, 68] Organisms normally nonpathogenic to a species can be made virulently pathogenic by cortisone treatment.[61] In a similar vein it was found that grouping markedly depressed the development of granulomas[27] and increased the severity of experimental trichinosis

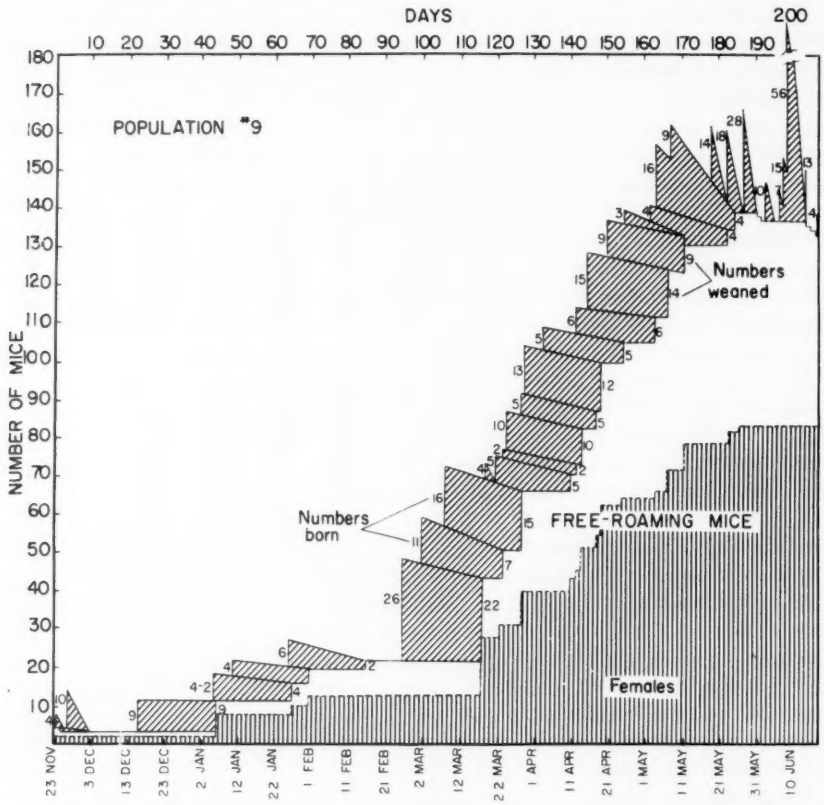

FIG. 4.—Plot of the growth of a freely growing, confined population of house mice. The vertical rises on the left of each cross-hatched area give the numbers born on a particular day, while those on the right show the number weaned. Note the increasing mortality of young as the population reaches an asymptotic level shown by the change in slopes of the upper lines on the cross-hatched areas. The total size of the population in terms of weaned mice is shown under the upper cross-hatched areas, while the number of females in the population is shown by the vertically barred portion of the "free-roaming mice." There are consistently more females than males. The growth form of this population is more or less typical.[16, 20]

in mice.[37] These effects parallel the effects of injecting cortisone and indicate that moderate increases in density may increase corticosteroid secretion sufficiently to suppress inflammation and resistance to trichinosis. Resistance to tetanus toxin, following tetanus toxoid, is similarly decreased by subsequent grouping[36] implying a suppression of antibody formation. These experiments demonstrate that increased density, presumably through inhibition of defense mechanisms by increased

endogenous adrenal corticoids, can increase mortality by reducing resistance to infectious disease. These results explain why epidemics, paradoxically often involving a variety of pathogens, are frequently but not always seen in natural populations of mammals at peak numbers.

The foregoing experiments demonstrate the following basic effects of increases in population density: increased adrenocortical activity, depression of reproductive functions with increasing size of the population, inhibition of growth, inhibition of sexual maturation, decreased resistance to disease, and inhibition of growth of nursing young with effects on subsequent generations, apparently through deficient lactation. However, these experiments were highly artificial and the question remains whether similar effects can be observed in populations which have been permitted to grow of their own accord, either in the laboratory or in the field.

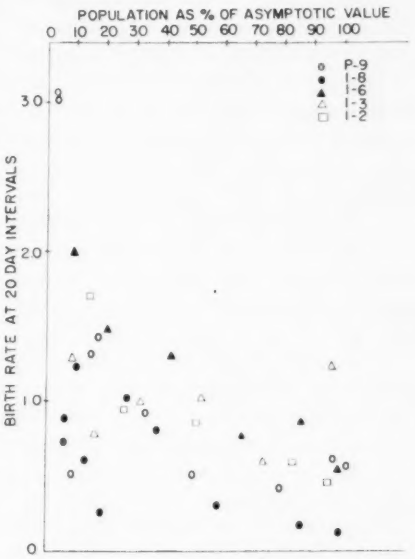

FIG. 5.—Birth rates for each 20-day interval plotted against the population size expressed as percentages of the asymptotic value for each of 5 experimental freely-growing populations. There is considerable variation in the birth rates when the populations are small, as one might anticipate with very small numbers, but curves become smoother as the population increases and sudden stepwise increases are lost in the total population. It is clear that the birth rate declines steadily with increasing population density. The same can be shown for the mortality rate of young between birth and weaning.[20]

In each of a series of experiments a few pairs of house mice were put into a large cage and permitted to breed at will in the presence of abundant food, water, nest material, and constant temperature and day length. The entire populations were sacrificed either at maximum size or at one half the anticipated maximum size.[16] Such populations will reach an asymptote in spite of excess of all tangible needs.[16, 63, 67] Figure 4 is a diagram of the growth of the most recent of these populations and is representative of the earlier similar experiments. The birth rate declines with increasing population size (Fig. 5). The rate of mortality from birth to weaning rises steadily with the increase in the population, due to a combination of factors including poor maternal behavior and nest interference, but probably most importantly to inhibition of lactation.[5, 16, 26, 54, 64] The decline in birth rate was found to be due to decreased fertility in mature females, inhibition of maturation, and significantly increased intrauterine mortality.[16] Similar declines in the birth rate have been observed in comparable experiments with freely growing confined populations of voles.[29]

If emigration is permitted from a population when it reaches levels which would completely suppress reproduction, it has been found that reproduction continues rather than being totally inhibited.[66] Similarly, according to Crowcroft and Rowe,[31]

reproduction recovered after it had been inhibited completely when emigration was permitted. They concluded that such densities, and therefore total inhibition of reproduction, will not occur in natural populations. The error of this conclusion is shown by the work of Evans,[42] Young *et al.*,[73] Davis,[33] and Kalela,[52] who cite examples of extreme densities obtained by microtines and house mice in the wild, with total suppression of reproduction. Also, as we have shown, social interactions are the governing factor rather than density in the literal sense. The obvious example of a failure of emigration to relieve inhibition of reproduction is the well-known periodic migrations of lemmings. Even under natural circumstances the ability to emigrate is not unlimited. Animals may move from a given local concentration but eventually the interstices between concentrations become fully occupied and the possibilities for emigration become restricted. There is evidence that small mammals normally are not distributed randomly or evenly. Furthermore local colonies behave as discrete populations until high densities are reached and, with expansions and emigration, the colonies coalesce and over a wide area the population behaves as one.[24] The densities of individual localized colonies may become quite high while the density of the entire population remains low. Finally, total inhibition of reproduction is not required to inhibit population growth and the data of Crowcroft and Rowe[31] show the inverse relationship between density and reproduction.

The organ weights of mice from the most recent freely growing experimental population of asymptotic size are given in Figure 6. These results essentially typify those from earlier experiments[16] except that in this case the ages of the individual mice are known, whereas previously they were not. Several facts stand out. Body weight was less in all but the oldest animals from the high population, and these differences are not due to variations in the amount of stored fat, but to actual differences in growth, as the amount of subcutaneous and depot fat, while present, was not impressive and was proportionately similar in amount in all but the youngest animals. The oldest mice were either those originally introduced or from the first litters born, and consequently probably were the dominant animals in the population. This statement is based on repeated observations, but it has not been systematically studied. There was an increase in adrenal weight, especially of the males, in all age groups. An increase in adrenal function consistent with the increase in weight is indicated by the greater involution of the thymus in animals from the high population versus their controls. The adrenals of female mice from populations of increased density show an increase in weight when compared to their controls (Fig. 6) or to those from groups of females only.[21] The adrenals of males increased on an average of 25 per cent and those of females about 15 per cent. The sudden decline in weight of the adrenals of both the control and experimental females at about 95 days of age (Fig. 6) reflects involution of the X-zone. Reproductive function (inhibition of gonadotrophin and androgen secretion) was suppressed, as shown by the lower weights of the testes, os penis, and (except for the oldest animals) the seminal vesicles. Earlier experiments showed that there was inhibition of maturation in the younger animals, which is also reflected here, although the degree of suppression is about equal in all animals less than 160 days of age. It is interesting that the form of the curve for the weights of the seminal vesicles of mice from the high population, with respect to age, is

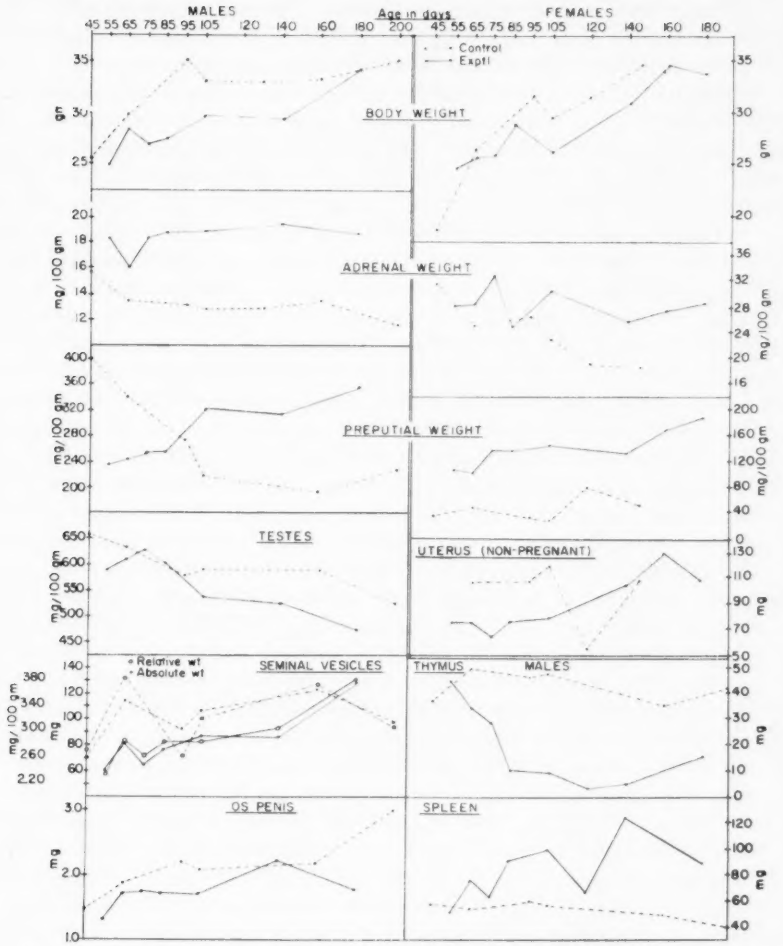

FIG. 6.—Organ weights plotted against age for male and female mice from the freely-growing population (No. 9) shown in Fig. 4. These results are typical for these populations[16] and can be interpreted similarly, but differ from earlier results in that the ages of the mice for this population were known, and therefore the organ weights can be plotted against age rather than weight. A suppression of growth in all mice but those from the first litters is clearly shown. These and, especially, originally introduced animals were dominant in the population. Note the greater preputial weight of males more than 95 days old compared to their controls, whereas the seminal vesicles were smaller in all except the mice from the first litters. These data suggest that one or more factors besides testosterone were responsible for stimulating preputial growth. A similar situation in the experimental females suggests that increased adrenal androgens, ACTH, or both stimulate greater preputial growth in the experimental animals of both sexes. The increased splenic weight in the experimental males is largely attributable to increased hematopoiesis.

the inverse of the adrenal curve and directly reflects the curve of the body weight, whether absolute weights or weights relative to body weight are used, suggesting that the same factors operating to suppress growth act equally to suppress androgen secretion. The reverse situation is true for the control animals, in which the curves

for the testes and seminal vesicles are parallel and reciprocally related to body weight and the os penis, suggesting that growth and reproductive function (at least as far as minor variations are concerned) are reciprocally related.

The preputial glands of mice from the high populations are at first smaller than in the control animals in this and preceding experiments and later (in the mice more than 85 days old) increase in weight to considerably greater than the control levels. In both control and experimental animals the development of these glands appears to parallel body weight much more closely than the testes or seminal vesicles; in fact, the forms of the curves for preputial weight appear to be the reciprocal of the curves for the latter, suggesting that endocrine factors other than testosterone are more important in controlling preputial growth. The preputials of females responded to increased density by an increase over control levels in this and in earlier experiments.[16] Adrenal androgens stimulate the preputials nearly as much as testosterone.[47] They also are stimulated by ACTH and growth hormone.[48, 49] These facts suggest that preputial growth in males first is inhibited by decreased testosterone secretion but later on, in older animals, increases in size over control levels, due to some sort of individual or combined action of ACTH, testosterone, adrenal androgens, and growth hormone. A combination of nearly normal amounts of testosterone and growth hormone (acting additively) with increased amounts of ACTH, and especially of adrenal androgens, seems to be the most likely suggestion, as physiological amounts of certain adrenal androgens, shown to stimulate the preputials, probably act synergistically with growth hormone.[48] This explanation fits the data from the females equally well. In the absence of testosterone and suppressed by estrogens, the preputials normally fail to develop but in this case may reflect suppression of estrogen with increased adrenal androgens, accounting for the increased preputial weights in mice from populations of increased density[16, 21] (Fig. 6). The secretion of adrenal androgens probably is controlled by ACTH, so that increased amounts of ACTH would be expected to increase the secretion of adrenal androgens, particularly when acting on hyperplastic cortical tissue.[4, 6, 38, 44, 46, 72] These facts suggest the means by which gonadotrophin secretion is suppressed in circumstances stimulating increased secretion of ACTH. The successful use of cortisone to suppress ACTH and, secondarily, the production of androgens, in cases of congenital adrenal hyperplasia, supports this suggestion.[6, 71] Furthermore, weak adrenal androgens have little stimulatory effect on the seminal vesicles, whereas their action on the preputials and os penis is quite marked and out of proportion to their effect on the seminal vesicles and other organs conventionally used to assay androgenic activity.[40, 47]

The results shown in Figure 6 along with those from earlier experiments[16] support the extension of conclusions derived from populations of fixed size to include freely growing populations of house mice.

The results from experiments with confined freely growing populations of voles (*Microtus*) coincide with those for populations of house mice.[20, 29, 54] Adrenal weight increases and reproductive function decreases with increased density to greater degrees and population growth terminates at much lower densities than for house mice under essentially identical experimental conditions.[20] Published research on confined freely growing populations apparently has been limited to these two species.

Natural populations of house mice, white-footed mice (*Peromyscus*), and voles (*Microtus, Clethrionomys*) provide data similar to those from freely growing confined populations. Adrenal weight in both sexes of urban Norway rats closely followed changes in relative density of the population,[20, 24] and reproduction shows the expected reciprocal relationship to the relative densities of the populations.[32] Furthermore, adrenal weight can be reduced about one-third by reducing the size of the populations by approximately one-half.[23] A similar reduction was observed for sika deer.[25] The growth of populations of rats can be altered by upsetting their social organization, as shown by experiments in which alien rats either were added to the population or else substituted for a like number which had been removed.[34] High stationary (relatively dense) populations promptly declined when alien rats were added to the populations. Increasing populations ceased growing when about 15 per cent of the population was removed and a similar number of aliens substituted. The importance of social relationships in populations of rats was demonstrated when it was shown that subordinate rats seldom raised young successfully and that dominant animals accounted for most of the reproduction.[9, 10] In another experiment, a rural population of rats was followed for several years by monthly trapping. The weights of the adrenals of both sexes in this population correlated closely with the size of the population and its ups and downs for the entire period, and pituitary weights correlated with adrenal weights.[20]

One of the important studies on natural populations was conducted by Kalela[52] on red-backed voles (*Clethrionomys*). A major factor in the value of this study was his ability to determine age of the animals. In populations of moderate density, most of the young of the year reached maturity, but in a period of higher density, maturation and growth were significantly inhibited, so that there was an accumulation of immature, relatively old animals throughout the summer. Males were more affected than females. The rate of population growth was very high earlier in the same season, but declined rapidly primarily due to the failure of the young animals to mature. As might be expected, the pregnancy rate also fell rapidly, mainly due to the decreasing numbers of mature individuals. These studies showed that reproductive function in immature animals is much more readily suppressed by increased density than it is in mature animals, although the latter were affected if the densities were great enough. In one area of extreme density, reproduction ceased altogether and no young reached maturity, but remained in a "subadult" condition. The following year, although the population had declined markedly, fecundity, pregnancy, and other measures of maturity remained the same as in the preceding year of high density, confirming that the effects of high density persist in the young, as suggested by Chitty[11] and shown in the laboratory.[12, 26] Kalela also demonstrated that food supply and climatic changes could not account for these effects.

These results were confirmed and extended in a five-year study of *Microtus montanus* in Montana by Adams, Bell, and Moore, who provided the author with the material collected from this population for further study.[20] A close association between adrenal weight and population size was found in these voles (Fig. 7), as well as evidence of a progressive inhibition of maturation with increasing density in each year. There was no evidence of inhibition of reproduction in the largest and presumably the oldest animals in this population (Fig. 8). Figure 8 shows

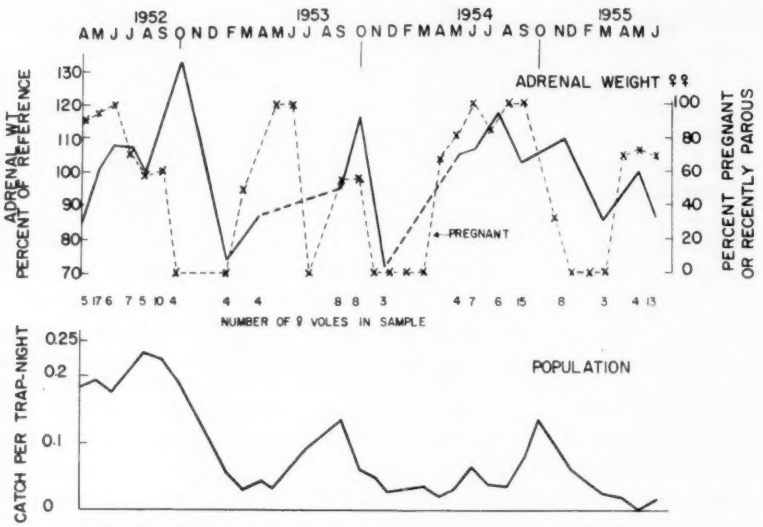

FIG. 7.—Adrenal weights expressed in percentages of reference values for each body weight category of female *Microtus montanus* from a population studied by Adams, Bell, and Moore. Note the close relationship between population size and adrenal weight annually and for the period of the study. Each year adrenal weight reaches a peak as the density has reached a peak and has begun to decline. Pregnancy rates decline with peak densities and subsequent declines in the population probably reflect the cessation of breeding with recruitment into the population. In the year of over-all maximum density, 1952, breeding began to decline in July and was not followed by a peak in the fall. In other years there was an autumnal peak in breeding with a peak in the population. The maximum adrenal weights seen also were associated with maximum densities in 1952. These data suggest that annual breeding cycles in this species are density-dependent. The reference adrenal weights were determined from a regression of adrenal weights on body weights with the animals divided into 10-gram weight intervals.

that as the population increased each year there was inhibition of testicular development affecting voles of progressively larger size, although never affecting those over 45 grams. Weight was used as a basis of comparison in spite of recognized shortcomings because, unlike *Clethrionomys*, there is no reliable way to determine age in this species. Inhibition of growth simultaneously with that of reproduction tends to minimize the actual magnitude of reproductive inhibition. However, even with this obfuscation, a direct relationship between the annual changes in density and the degree of inhibition of reproduction is apparent. The rapid decline in mean testicular weight and in prevalence of pregnancy in midsummer in 1952 (a year of peak density) with no rise again in the autumn contrasts sharply with the declines in these indices of reproductive activity in September–October of the succeeding years. That the so-called seasonal (autumnal) decline in reproductive function is not due to simple seasonal regression of the gonads is shown by several facts. First, the degree of development of the baculum (os penis) provided a reliable means of determining whether a male vole had been mature and subsequently regressed or never had matured. In no case did one of these animals with small, immature-appearing testes have a baculum indicating prior maturation with subsequent regression. Reference to the baculum eventually enabled us to use the appearance of the testes for determining whether the testes

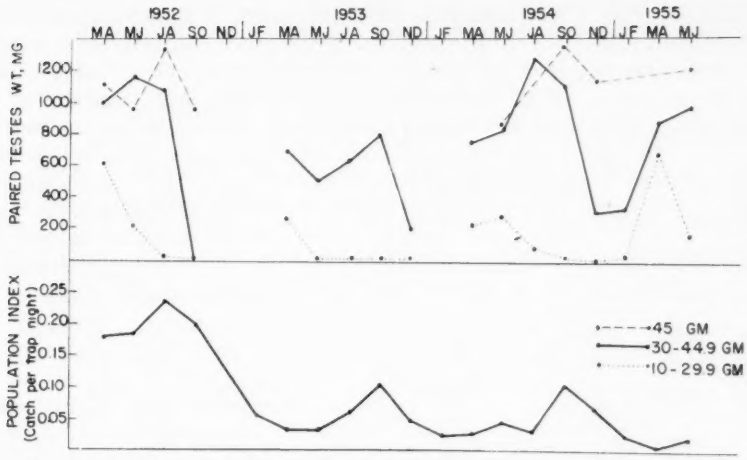

FIG. 8.—Data from male *Microtus montanus* from the same population as in Fig. 7. Mean testicular weights for three categories of body-weight are shown. These weight categories were selected on the basis that the data for testicular weight separated naturally into these three groups when testes weight was plotted against body weight for each 2-month period of the year. Note the absence of seasonal testicular regression in animals over 45 grams. The absence of animals of this weight from the population in 1953 evidently reflects severe inhibition of growth in animals born in late 1952 or early 1953 and the total disappearance of any large animals present in 1952. Inability to determine the age of these animals handicaps the interpretation of these data. However, it is likely that each year, as density increases, each succeeding generation is more seriously affected. Such population peaks coincide with a decline in the testicular weight of animals weighing 30–44.9 grams whereas smaller animals are affected earlier and at lower densities. These data coincide with the pregnancy figures for females and further suggest that the annual breeding cycle is density-dependent in this species. Increased reproductive activity precedes population peaks and begins to decline sooner. It is generally assumed that the annual decline in testes size usually seen in the late fall is due to meteorological or physical factors in the environment. The failure of the testes of the largest animals, presumably dominant and also born earlier in the year, is convincing evidence against this interpretation. From these data it is apparent that the decline in testicular size in animals less than 45 grams is due to progressive inhibition of maturation with increased density rather than to environmental factors. A study of the bacula (*os penis*) of these animals confirms that they never had reached maturity rather than having regressed.

were regressed or had never matured. Males weighing more than 45 grams (and therefore probably ones that had reached maturity early in the spring) showed no evidence of testicular regression in late fall or winter. In a subsequent study elsewhere on *Microtus pennsylvanicus* some large males showed no testicular regression in late fall or early winter, although others did (Fig. 9). The fact that there are individuals failing to exhibit regression rules out climatic or meteorological factors as important causes of reproductive regression. Finally, these results help explain reports that breeding sometimes takes place throughout the winter in voles, especially prior to attaining peak densities,[2, 45] and would appear to answer the question raised by Dice[39] in this regard. We have concluded, from these data and a considerable amount of less direct evidence, that annual seasonal changes in reproduction in *Microtus* (probably also in other species of voles) are largely density-dependent and that annual changes in the sizes of their populations result from changes in reproductive function in the presence of a more or less constantly

high mortality rate. The primary mechanism appears to be a progressive inhibition of maturation and growth of the members of the population as density increases, those from later births in each breeding season being successively more affected than the preceding. Voles which have failed to mature apparently over-winter in this immature condition, following birth during the annual late summer and autumn rise in density. Growth and maturation then resume in the late winter, after densities have reached low levels, possibly abetted by meteorologic factors at this time—for example, increasing day length. In general, meteorologic and climatic factors apparently play a minor role in controlling the annual cycles of reproduction and density in voles. The year-to-year inequalities of peaks of density might be questioned except that, even in years of low general abundance, localized foci or colonies attain relatively high densities[24] and that indices of the size of the population usually are obtained from greater areas than are occupied by such local concentrations. Also, the suppression of reproduction and inhibition of growth clearly are far greater and occur considerably earlier in years of maximum general density than in years of moderate or low general densities (Figs. 7, 8, and 11 and ref. 52).

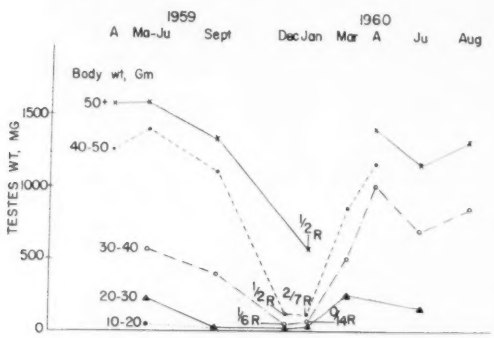

FIG. 9.—Mean weights of the testes plotted against time of year for each 10 gram body weight interval of male *Microtus pennsylvanicus* from a population on the Letterkenny Army Ordnance Depot near Chambersburg, Pennsylvania. These results are essentially similar to those shown in Fig. 8 for the same species from Montana except that in this case there are some animals showing testicular regression in December and January. The fractions preceding an R at these points give the number of voles with evidence of testicular regression out of the total sample size. The testes of the majority of males did not regress, but failed to mature. Of the two animals in the 50+ gram weight group for December–January, one animal had unregressed testes and the other totally regressed, so that if the former were plotted by itself, there would be little change in testicular weight with season in animals of this size. The existence of one animal with mature testes and without evidence of regression at this time of year casts the whole idea of climatic control of the reproductive cycle of this species into doubt. Additional examples of mature unregressed testes in late fall and early winter have been collected recently but too late to be included in the figure. However, seasonal climatic factors may serve to synchronize the breeding cycle. It would appear that the seasonal decline in testicular weight of samples from this population is due to a combination of a progressively more severe inhibition of maturation with increased density, coupled with the short life expectancy of this species, so that the mature and older animals disappear from the population leaving a preponderance of younger animals, whose maturation has been inhibited. Low densities in winter may permit increasing day length to stimulate a resurgence of reproductive activity.

These conclusions with regard to voles are supported by these studies on *Microtus* and Kalela's results[52] even though partial testicular regression in large voles was occasionally seen in a latter study on *Microtus* (Fig. 9). However, the fact that even a few large voles show no evidence of regression makes an important regulatory role of seasonal climatic factors unlikely. A companion study on *Peromyscus maniculatus bairdii* revealed a quite different situation. The annual reproduction cycle appears to be largely independent of density effects in this species, as all mature males undergo testicular regression in late fall (Fig. 10). Nevertheless, density effects still may play a

role (Figs. 10, 11), although undoubtedly less importantly than for *Microtus*. However, even in *Peromyscus* there is evidence of inhibition of maturation in the animals born during annual peaks of density (Figs. 10, 11) and adrenocortical responses to changes in density as seen in other species.[20]

The results of these and other studies on rodents and lagomorphs, reviewed in much greater detail elsewhere,[20, 22] agree with those from the laboratory, and indicate that the conclusions derived from confined populations with regard to the feed-back physiologic regulation of population growth can be extended to include natural populations of rodents.

The question often arises as to whether or not the conclusions derived from studies of rodents and lagomorphs can be extended to other species from other orders of

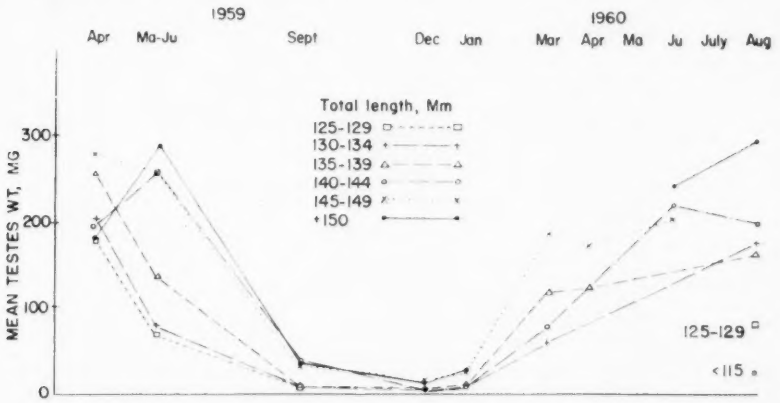

FIG. 10.—Mean testicular weights of male *Peromyscus maniculatus bairdii* from a population on the same area as the *Microtus* population shown in Fig. 9, except that the testicular weights are plotted against categories of lengths of the animals rather than weights. The same pattern of failure to mature sexually is seen in this species as in *Microtus*, but in addition there is seasonal regression of the testes of mature animals in the autumn and winter. Of the males with a total length of 140 mm or more in the December and January samples, 9 had regressed testes and had been mature previously, and 13 had never matured. No previously mature mice were collected in March. On the bases of these data environmental regulation of the seasonal regulation of reproduction cannot be ruled out, but that this species is also subject to inhibition of maturation associated with increased density is also indicated by the data from April, May, and June.

mammals. A recent study has shown that these same phenomena occur in sika deer (*Cervus nippon*).[25] A population of sika deer reached a maximum density of about one per acre and subsequently experienced a mass mortality which reduced the herd by approximately three-fifths. Growth was inhibited as much as 40 per cent in deer born during the period of maximum density preceding the die-off. Deer born earlier showed less inhibition of growth, while those born afterwards grew normally and reached greater size. The decline in density was accompanied by a proportional reduction in adrenal weight. At the same time, hepatitis first appeared, apparently as a result of decreased resistance accompanying the response to high density, which is still present in the population. Glomerulonephritis also was found in these deer during the period of maximum density but a year later showed signs of resolution in the involved animals. This disease

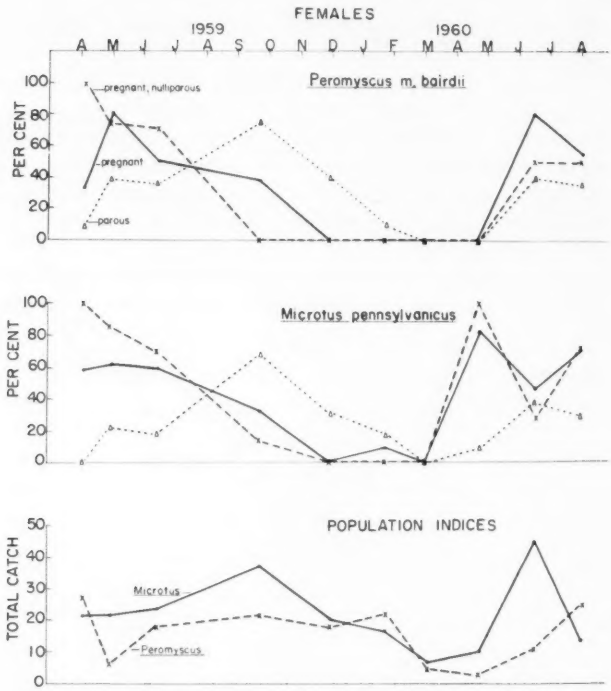

FIG. 11.—Data on female reproductive performance in relation to population density for *Microtus pennsylvanicus* and *Peromyscus maniculatus bairdii* from the same populations shown in Figs. 9 and 10. The per cent pregnant (pregnant, solid line), the per cent of these pregnant for the first time (pregnant, nulliparous, broken line) and the per cent parous (dotted line), whether pregnant or not, are given for each sample. The relative population size as given in terms of total catch for the first night for each sampling period is shown in the lowest portion. The data for the two species are remarkably similar for 1959 when their populations were similar. However, in 1960, there was a marked increase in the populations of *Microtus* in June and July, apparently produced by a highly successful breeding season earlier in the spring; nevertheless, the per cent pregnant then declined sharply as the population rose, suggesting density-dependent inhibition of reproduction similar to that noted for the same species from Montana (Fig. 7). On the other hand, the prevalence of pregnancy in *Peromyscus* was at its maximum a month later and then began to decline. The differences in the two populations reflect these differences in prevalence of pregnancy at different times in 1960 in contrast to their similarity in 1959.

apparently did not contribute materially to mortality of the deer. Mortality evidently resulted from shock following severe metabolic disturbances, probably as a result of prolonged adrenocortical hyperactivity, judging from histological material. There was no evidence of infection, starvation, or other obvious cause to explain the mass mortality. Deer, as well as rodents and lagomorphs, respond to increased density with increased adrenocortical activity and inhibition of growth. Sufficient data were not available to assess effects on maturation.

The effects of increased density on maturation and reproduction present an intriguing problem, as the mechanisms which reduce the secretion of gonadotrophins

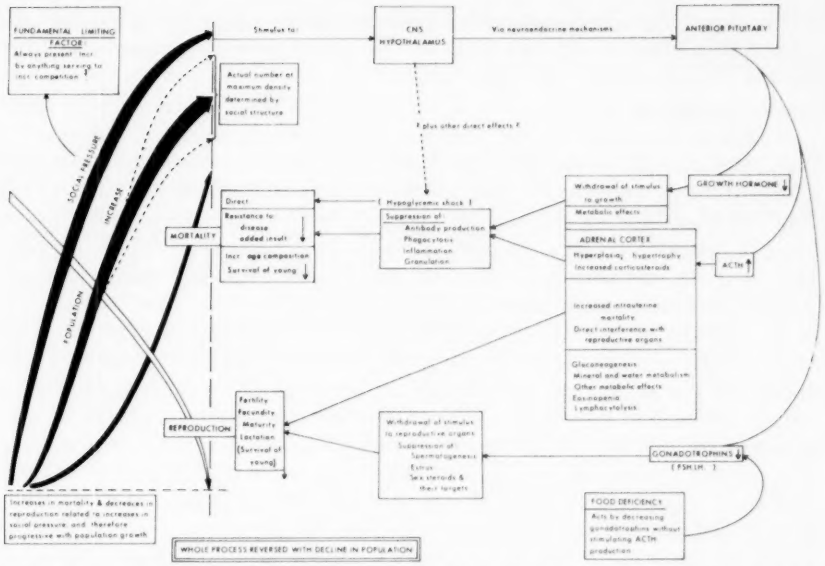

FIG. 12.—A graphical summary of the means by which density related-social pressures operate through physiological mechanisms to regulate population growth. The arrows on the left indicate that as population increases, social pressure increases and increases the magnitude to the physiological effects. The dotted lines above and below the arrow for population increase indicate that there may be considerable variation in the actual number of animals required to produce a given level of "social pressure" depending on the behavioral composition of the population, such as the aggressiveness of the dominant animals, clarity of definition of rank differences, and probably other unknown behavioral factors. Environmental factors operating to increase competition will in effect increase the social density or, in numerical terms, will serve to lower the upper asymptote of population growth in terms of numbers. Arrows indicate the mortality increases and reproduction decreases as the population increases, and the mechanisms by which these are achieved are shown. It must be remembered that this scheme represents average changes for the population as a whole and that great variations in response of individuals with respect to social rank will be observed, although the greater the density, the greater the proportion of the animals affected and the greater the severity of the effects.

This diagram certainly cannot be complete, but it can only be completed gradually as various physiological responses to behavioral and various environmental factors are studied in detail in relation to changes in density. Probably the least understood at the present time are the behavioral and social factors.

are unknown. Any explanation must account for the more pronounced effects on immature as compared to mature animals from the same population. Some of these effects probably are due to differences in social rank, the younger animals being more subordinate. However, this explanation does not account entirely for the strikingly greater effects on the young. Earlier work in which gonadotrophins were suppressed by injecting steroids[7, 8] showed that suppression can be accomplished in immature rats with about one-tenth the dose of steroid required to achieve the same effect in mature animals. This seems to be an important lead to possible mechanisms and, when combined with results discussed earlier, strongly suggests that increased secretions of adrenal androgens, in response to increased levels of ACTH, may be of importance in inhibiting reproductive function with increased density.

Summary and Conclusions.—Thus, it seems that a complex system of feed-backs

which respond to socio-psychological pressures have evolved which regulate and control population growth and keep the population within environmental limits but not without pronounced fluctuations. These feed-backs manifest their effects largely through the reproductive system and secondarily by increasing mortality. Our present knowledge of the physiologic and socio-psychological regulation of population growth is summarized graphically in Figure 12. Selection must operate largely through the dominant individuals in a population, as in most cases these bear the major load of reproduction and, furthermore, their young are the most likely to survive and reproduce normally. The growing evidence for the profound prolonged effects of increased density on offspring born during periods of increased density has important implications on the selection process as well as for the growth of populations, especially in determining the intervals between major peaks of density. The fact that these physiological reactions are brought about largely by behavioral phenomena represents progress in the evolution of independence from environmental fluctuations, as contrasted to direct dependence on food, climatic changes, and other factors of the environment, for the regulation of reproduction and numbers by eliciting directly these physiologic responses. A brief summary of evidence has been presented which indicates that even the annual cycles of reproduction and numbers of *Microtus* are largely density-dependent. Therefore mammalian populations, at least of the species so far studied sufficiently (*Mus*, *Rattus*, *Microtus*, *Clethrionomys*, *Marmota*, *Oryctolagus*, *Lepus*, *Sylvilagus*, *Cervus*, and as Dr. Ratcliffe will show, *Myocastor*), regulate the sizes of their populations largely through internal behavioral-physiological interactions. Insofar as experiments are analogous and permit conclusions, dogs, guinea pigs, monkeys, and man respond similarly to increased numbers, at least in terms of increased secretion of adrenocortical steroids. When environmental factors do exert controlling effects, they probably do so largely by altering the social or competitive situation and thereby shifting social pressures up or down, rather than by acting directly. The evolution of the social regulation of population growth could be considered a marked developmental advance over direct environmental regulation, and coincides with the greater development and importance of the higher central nervous system and warm-bloodedness in mammals in contrast to the lower animals. Finally, since the survival and evolution of a species must depend on reproduction by dominant individuals, the selective process would seem to be operating in a direction to increase the importance of behavioral adaptation.

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*EVOLUTION AND INTEGRATION OF MECHANISMS THAT REGULATE POPULATION GROWTH**

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Present evidence provides unequivocal support for the thesis that the growth of mammalian populations is basically regulated by physiologic, especially endocrine, responses to population density.[1] Included in this thesis is the concept that interactions between mammals in the same population (intraspecific competition, social interaction, etc.) are intensified as density increases. At its lower levels social interaction may promote the breeding and productivity needed to maintain a population or to allow it to grow. But high productivity and a growing population must also increase the level of social interaction, which shifts endocrine adaptive mechanisms (raises adrenocorticotrophins and diminishes gonadotrophins) to reduce productivity and increase mortality.[1]

The earlier research on endocrine adaptive mechanisms was conducted primarily with laboratory populations of mice; however, there is now considerable evidence to indicate a density-dependent increase in the activity of physiologic adaptive mechanisms in natural populations of Norway rats, voles (*Microtus, Clethrionomys, Dicrostonyx, Lemmus, Ondatra*), hares (*Lepus*), rabbits (*Oryctolagus*), and white-footed mice (*Peromyscus*).[1] A relationship between population density and adrenocortical activity has been demonstrated for Norway rats, meadow voles (*Microtus pennsylvanicus*) and white-footed mice (*Peromyscus leucopus*).[1] An inverse relationship between density and reproductive function exists for Norway rats, red-backed voles (*Clethrionomys*), meadow voles (*Microtus agrestis, M. mon-*

tanus, M. californicus, M. pennsylvanicus), muskrats (*Ondatra*), cotton rats (*Sigmodon*), and hares (*Lepus*).[1]

Even so, some ecologists, although they do not deny the importance of social interactions in regulating numbers of confined populations of small mammals, question the significance of these factors in natural populations. If intraspecific competition is the basic stimulus to physiologic responses that regulate the numbers of animals in relation to the environment, then the stimulus must operate through at least one of the three forces of population growth: (1) reproduction, the force for increase; (2) mortality, the force for decrease; and (3) movement, a force for either increase or decrease, depending on whether animals move into the population from the exterior or move away from the space limits of the population. Therefore, a summary of the results of a comprehensive study of natural populations of woodchucks (*Marmota monax*) in southcentral Pennsylvania may be of interest at this time because it illustrates the principle that natality, mortality, and movement all vary with changes in population size and composition.[2, 3]

Study of Woodchuck Populations.—Woodchucks belong to the family Sciuridae of the order Rodentia. They have a combination of the fossorial and terrestrial adaptations and they hibernate during the colder months of the year. Their principal foods are grasses and the leaves of clover, alfalfa, plantains, and various perennials.

Certain characteristics of these large rodents make them highly useful subjects for studies of population dynamics. They are relatively easy to trap, thus numbers can be determined by various routine methods of censusing. Females have no more than one litter of young each year, which in southern Pennsylvania are born during April. Thus, populations go through annual cycles in which numbers increase abruptly after the young are born and then decline gradually until the next breeding season. Reproductive performance can be determined by collecting and examining a representative sample of the females during the breeding season each year.[4] At this time percentage pregnancy, reproductive rates, and birth rates can be calculated by counting corpora lutea, viable and resorbing embryos and fetuses, and placental scars.[4] Mortality rates can be calculated indirectly by trapping, marking, and releasing a number of animals in a population one year and then determining how many disappear by the following year.[3] The extent of movements can be determined by noting the locations at which marked animals are trapped.[3] Thus, each of the three forces of population growth can be measured and related to population size and composition.

The study of woodchucks was conducted on the Letterkenny Army Ordnance Depot which covers nearly 20,000 acres. The investigation was limited to about half of this area, where topography and ground vegetation were highly uniform. Here woodchucks were at least tenfold more numerous than in the intensively cultivated farmlands adjacent, at times approaching densities of one animal per acre.[3]

The section of the depot used in this study is divided into 15 rectangular units of 300 to 600 acres. These are separated one from another by zones of cultivated fields 1,300 to 1,500 feet wide. Two of these units were selected and an attempt was made to create a sparse population on one area of 600 acres while maintaining a dense population on the other area of 535 acres. The zones of cultivated fields between these smaller study areas, although not barriers to movement, separated

the populations geographically. One of the objectives of the study was to compare reproduction, mortality, and movements in populations at different densities. The food supply was always in excess of the needs of the animals, as from 90 to 95 per cent of the land consisted of meadows of grasses (especially Kentucky Bluegrass, *Poa pratensis*), clovers, plantains, and perennial weeds.

Yearling and adult woodchucks were removed from one area (exploited) at an average rate of 30 per month for eight months, February through September, of each year in 1957 and 1958, while only as many animals as were needed to study reproduction each spring were removed from the adjacent reference area (protected). Animals were not eliminated from the exploited population until they were at least a year old, thus the program was expected to decrease density and mean age of the members of the population concomitantly.

The two populations were of equal density in the spring of 1957 at the beginning of the study, but although more than 500 woodchucks were removed from one area in a two-year period (about five times as many as were taken from the reference area), censuses each spring and again each fall in 1957 and in 1958 did not demonstrate any significant differences in the densities of the two populations.

The breeding population in the protected study area each spring contained 70 per cent adults and 30 per cent yearlings (young born the previous year). This population was relatively stable; that is, it contained about the same number of animals each spring. Under these circumstances 30 per cent of the adults would die each year and be replaced by reproduction. The exploited population contained just as many animals per acre each spring as did the protected population, but the proportion of adults in this population each spring changed from 70 per cent in 1957 to 57 per cent in 1958 to 39 per cent in 1959 (Table 1). A change in the age composition of this population was expected; however, one must account for the fact that although animals were removed continuously the density did not drop below that in the reference area. The most likely compensatory factors were as follows: (1) more young were born following the removal of the older woodchucks, (2) more young animals survived than heretofore, or (3) young animals moved into population from the exterior as the older animals were eliminated.

Evidence for increased reproduction following reduction in numbers is presented in Table 1. Although the mean number of corpora lutea per pregnant female, which is a measure of the ovulation rate,[4] and the mean number of implantations (an embryo, a fetus, or a placental scar is evidence of an implantation) per pregnant female declined from 1957 to 1959, the birth rate was highest in 1958. The ovulation rate, average litter size, etc., increases with parity in woodchucks,[4] thus the decline in ovulations and implantations must have been a reflection of a lower mean age of the females, as this population contained proportionately more yearling females and fewer adult females each year. Prenatal losses were less following the removal of adult woodchucks from this population; hence, the average litter size (or the average number of live young produced per pregnant female)[4] was higher in 1958 than in 1957. There was definite improvement in reproduction because a higher proportion of the yearling females became pregnant and because intrauterine mortality of embryos and fetuses was considerably less. An increase in the percentage of yearling females pregnant from 20 per cent in 1957 to 56 per cent in 1958 and 42 per cent in 1959 is significant because experiments with confined

populations of mice show that crowding (increased density) delays the attainment of sexual maturity in mice to a marked degree.[1] Crowding also increased intrauterine mortality in laboratory mice.[1]

Reproduction was lowest in 1959, but now only 40 per cent of the breeding females were more than a year old and apparently no more than 50 to 60 per cent of the yearling females become pregnant even when social pressures are reduced. Therefore, a point must have been reached when the average age of the females was too low to allow maximum production.

A relative measure of mortality was made by calculating the rate at which woodchucks disappeared from the populations. A certain number of the animals were marked and released during one year, then the percentage that was recovered in each subsequent year was calculated. This method did not allow a comparison of the rates of disappearance from different populations, as the percentage of animals recovered is directly proportional to the effort made to recover them. Although considerable effort was made to recover marked animals in all study areas, the effort was not the same in each one. The value of this method lies in comparing the rate of disappearance of different age classes or between the sexes

TABLE 1

IMPROVEMENT IN REPRODUCTION AS WOODCHUCKS WERE REMOVED FROM THE POPULATION*

	Status of population		
	1957	1958	1959
Means per pregnant female	Dense	Removal of animals	Removal continued
Mean corpora lutea	5.46 (71)	5.27 (52)	4.71 (31)
Mean implantations	4.67 (76)	4.34 (83)	4.03 (21)
Mean embryo loss	−1.25 (32)	−0.23 (31)	−0.39 (23)
Mean litter size	3.42	4.11	3.64
Total per cent ♀ ♀ pregnant	75 (101)	79 (106)	61 (51)
Per cent yearlings in population	30 (122)	43 (124)	61 (161)
Per cent yearlings ♀ ♀ pregnant	20 (31)	56 (50)	42 (33)
Per cent adult ♀ ♀ pregnant	100 (70)	100 (56)	94 (18)
Birth rate	1.29	1.63	1.11

* Numbers of animals examined to obtain each value are given in parentheses.

within each area, as all of the animals within an area were subjected to equal trapping pressure.

The numbers of woodchucks marked and released in each area and the proportions of these woodchucks that were recovered subsequently during the next year are listed in Table 2. Animals are grouped according to age. Those marked as yearlings or adults are referred to as old woodchucks, while those first marked as young of the year are referred to as young woodchucks. Woodchucks marked as young of the year were recovered as yearlings the next year, while those marked as yearlings and adults were two years old or older when recovered.

More than 50 per cent of the old woodchucks marked and released in the area from which few animals were removed compared with less than 25 per cent of the young woodchucks marked and released there were subsequently recovered ($P < 0.01$). In contrast the proportion of young recovered from the exploited population was not significantly different from the proportion of older animals recovered from this population ($P > 0.30$). Normally the mortality rate of the young in a dense population is much higher than the rate among the older animals. For example, a differentially higher rate of mortality for younger animals has been observed in populations of prairie dogs by King[5] and in populations of snowshoe

hares by Green and Evans.[6] The significant point is that young woodchucks survived as well or nearly as well as did the older woodchucks in the population after a portion of the older animals had been removed. Thus, increased survival of the young animals to approximate that of the older animals is clearly indicated as a second compensation for a reduction in density.

The distances that woodchucks moved between successive points of capture were recorded during this study to determine if movements were related in any way to population density. The young of the year moved over a much larger area than did the adult woodchucks; also a portion of the young born in each study area moved away or emigrated across the boundary zones into adjacent areas. With two exceptions, none of the older woodchucks of either sex in any area moved more than 2,600 feet between captures, and 95 per cent of their movements were confined to 2,000 feet (based on an analysis of 532 movements). From 23 to 33 per cent of the young of the year marked and released in an area were recovered during the following year in the same area. The largest percentage of these young woodchucks disappeared from the populations.

Significantly more young woodchucks emigrated from the protected area than

TABLE 2

THE PROPORTIONS OF MARKED WOODCHUCKS THAT WERE RECOVERED DURING THE YEAR AFTER THEY WERE RELEASED

	Exploited population		Protected population	
	Old	Young	Old	Young
Marked and released one year	48	154	159	101
Recovered the next	20	50	85	23
Per cent recovered	41.7	32.5	53.5	22.8

from the exploited area, which would indicate that the movement of young woodchucks was influenced by population density. Only four woodchucks marked and released initially (1.9% of 154) in the exploited area were recaptured subsequently in other study areas, while 18 woodchucks marked initially (8.4% of 215) outside of this area were subsequently recovered within its boundaries. Apparently young woodchucks emigrated from dense populations on the exterior and settled in the area from which so many adult woodchucks had been removed. The implication is not that movements were directed to this area because the population density was low, but rather that young woodchucks moved into the area at random and remained there because population pressures were low. A tendency for young animals to move into the population as the older animals were eliminated would be a third compensation for a reduction in density.

The results of this study illustrate the principle that natality, mortality, and movement, as forces of population growth, respond to changes in density, which is further evidence for the existence of a density-dependent adaptive mechanism. In areas that were maintained as dense populations (i.e. relatively few animals were removed) only 20 per cent of the yearling females became pregnant and intrauterine mortality was high (an average of 1.25 embryos and fetuses lost per pregnancy). Few young animals survived to maturity and many emigrated from these populations. Clearly these responses would limit population growth. On the other hand, following the elimination of a portion of the older animals from an area, reproduction improved, more young woodchucks survived and remained in the

area, and at the same time several young woodchucks moved into the population. In turn these responses would promote growth of the population.

The summation of these density-dependent responses would cause densities to level out over relatively large areas, which would explain why it was not possible to maintain different levels of density in areas not widely separated. Age compositions were not the same in the two areas, because of the tendency for the older animals to remain in a home area once they were established there.

The data on movements of woodchucks give, perhaps, the most important single clue to the role of social factors in regulating population growth. Records include 790 movements of woodchucks from one trap site to another. The distance traveled between successive points of capture for adult woodchucks averaged 410 feet; the males traveled over a slightly greater area than did the females. In comparison, the young of the year after weaning moved an average of 1,000 feet (maximum 10,000 feet) from one point of capture to another. Young woodchucks were apparently moving about in search of a home area, home region, or whatever term is applicable. On the other hand, the relative lack of movement on the part of the older animals is basic to the concept that social pressures increase in a growing population. Once a woodchuck becomes established in a certain location it apparently remains there for long periods of time. Trapping records show that animals have remained in one place (a home area) for as long as three years. The tendency for an animal to establish itself in a limited area and remain there for long periods of time is significant, I think, because there must be several social or behavioral interactions involved to allow such an arrangement. Animals that do not succeed in establishing a home area apparently do not survive, since there are no records of extensive movements once an animal was more than a year old. Home areas are apparently maintained by a sort of mutual tolerance on the part of neighbors in the population. Increased density must increase social pressures and make it more difficult for neighbors to maintain their social position toward one another. Young animals, once they are weaned and leave the maternal den, also must find a place in this social structure. Thus, the evidence indicates that when competition for social position is intensified by increased density or by certain changes in population structure (i.e. a surplus of males during the breeding season[3]) endocrine adaptive mechanisms are activated which reduce productivity and increase mortality. The subordinate members of the population are undoubtedly the most affected by increased social competition.

This study provides additional evidence from another species for the thesis that intraspecific competition is the basic stimulus to physiologic responses that regulate numbers of mammals. Responses in reproduction, mortality, and movement were related to changes in density and were integrated to the extent that the populations were able to adjust to manipulations of density. The level of social interaction could not be measured directly in each situation, but the results of this study combined with those from the earlier carefully controlled laboratory experiments allow one to be reasonably certain that basically reproduction, mortality, and movement varied in response to changes in the levels of intraspecific competition.

Natural Selection of Social Systems.—Christian[1] sees social pressure hypothetically as the fundamental growth-regulating and growth-limiting factor in all mammalian populations and the factor to which physiologic adaptive mechanisms

respond. However, because this factor is always present and always operative, it does not necessarily imply that it is always the factor which limits population growth. Environmental factors probably operate through this mechanism by increasing or decreasing social pressures, although conclusive evidence for this statement is presently lacking.

The term "social" has been used in a general sense to include "all groupings of individuals which are sufficiently integrated so that natural selection can act on them as units."[7] On this basis natural selection could act on adaptive density control mechanisms that are intrinsic parts of a population's social structure. Thus natality, mortality, and dispersal may all vary in relation to density and keep a population within the bounds of its environment. A flexible mechanism of population control would allow a population to utilize more fully the capacities of the environment, but at the same time prevent the overutilization of the food supply or the destruction of shelter. Stated in another way, social systems that elicit physiologic adaptive responses in reproduction, mortality, and movement as population density increases may be selected by evolutionary processes because they allow a population better adaptation to the environment.

* Supported in part by Grants H-4759 and H-2489, National Institutes of Health, U.S. Public Health Service.

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*DISCUSSION OF THE PAPERS BY CHRISTIAN AND SNYDER**

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The records of the Penrose Research Laboratory of the Zoological Society of Philadelphia have provided considerable evidence in support of the thesis that social interactions operate through endocrine adaptive mechanisms to limit population density. Thus, I may cite failures in breeding and lactation, inadequate development of offspring, and increased susceptibility to microorganisms in groups of mammals and birds on exhibition in the Philadelphia Zoo. These failures have

been associated with increased numbers in each group. The records of this laboratory also suggest that increasing population density has been a major factor in recent increases in frequency and severity of coronary arteriosclerosis of both mammals and birds in the Philadelphia Zoological Garden.[1, 2]

These manifestations of social interactions among the animals of this zoo (and perhaps their recognition) has depended upon the following combination of circumstances: first, between 1930 and 1946 economic and military considerations that caused a reduction in the number of animals on exhibition; second, the traditional and often inadequate rations then common to zoological gardens were re-

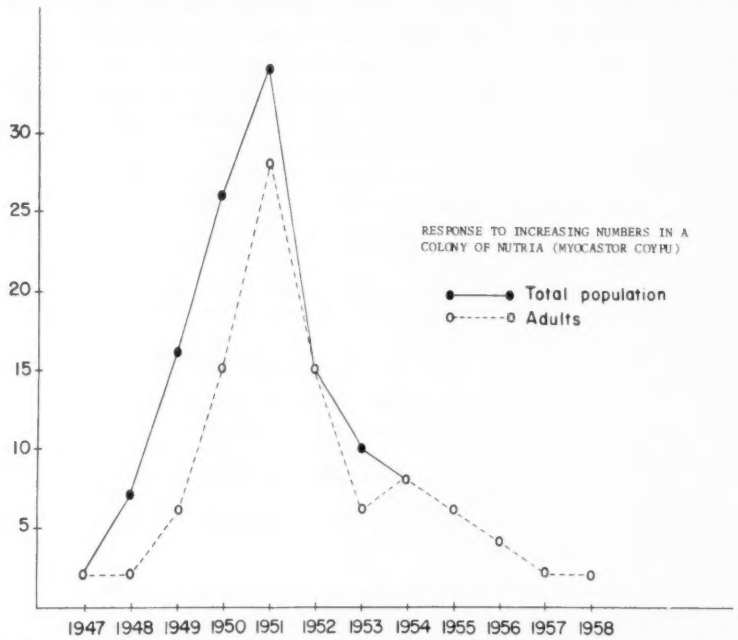

FIG. 1.—Records for a confined population of nutria (*Myocastor coypu*) over a period of 12 years. The total population for each year is the number of adults plus any young that survived more than one month. All young that survived one month and from one calendar year to the next are listed as adults. Note the abrupt decline in numbers and reproductive failures after 1951.

placed here in 1935 by controlled diets, which have been continued; and third, within the decade after 1946 the numbers of mammals and birds in the exhibition groups were again increased to equal or exceed numbers present prior to 1930.

Improved nutrition for this animal collection was followed by pronounced and anticipated increases in longevity and productivity (successful breeding and adequate development of offspring). However, the ready market of the decade prior to 1946 easily absorbed surplus stock and limited or prevented increases in the breeding groups. Thus our records of adequately nourished animals in the Philadelphia Zoo span two periods: before and after significant increases in population density.

A striking response to an increased population is illustrated by the values that were used to construct the graphs of Figure 1. These are the records for a group of breeding nutria (*Myocastor coypu*) during a period of 12 years. This group was started with an adult male and female which were purchased in November, 1947, and established in an outdoor pen. This pen is roughly circular, about 10 meters in diameter, and contains a pool and stone shelter.

Productivity in this colony is indicated by the graph of Figure 1 for the total population, and for "adult" animals. Offspring that survived more than one month are included in the total population. Any that survived for more than one month and from one calendar year to the next are listed as "adults." Thus Figure 1 shows that this group increased rapidly through 1951. Nevertheless, more than half of all offspring after 1949 died (apparently of lactation failure) within a month of birth. Only 10 offspring were produced after 1951 (in 1953). Four of these survived to adult age. However, none developed adequately nor reproduced. These constituted the colony after 1955.

Less disastrous responses to unchecked increases in breeding groups may be illustrated by the records for the tahrs (*Hemitragus jemlahicus*) and aoudads (*Ammotragus lervia*). Increasing density in these groups reached its maximum in 1956–1957, at which time all offspring (about 25 animals) were deserted by the females at birth. Few of these responded to artificial feeding. Accordingly, both groups were reduced by about two-thirds during the autumn of 1957. All offspring in 1958 were suckled by their mothers and all developed adequately. Thus the densities of these and other breeding groups will continue to be limited.

Similar responses have been observed in groups of kob antelopes (*Adenota kob-thomasi*), barasingha deer (*Rucervus duvaucelii*) and white-tailed deer (*Odocoileus virginianus*). However, the young of these deer have not been deserted so often at birth. Instead, they seem to lack a normal capacity for growth in the herd (reduced output of growth hormone?), but, if transferred, with others of the same age or alone, to isolated pens within two to three months of birth, they usually develop adequately.

Increased susceptibility to a potentially pathogenic microorganism as a response to numbers in the group is suggested by our records for the Macropodidae. Thus from 1936 to 1946 this family was represented by 20 animals, and none of nine offspring died of failure in lactation. From 1946 to 1956 this family was represented by 70 animals, among which were 10 deaths (half of all offspring) from failures in lactation. This family also lost 10 juveniles and young adults from toxoplasmosis within this decade.

Animals of the family Macropodidae are known to carry *Toxoplasma* (sp.?) as an apparently harmless parasite. However, our first example of this organism as a pathogen for animals of this family did not develop until 1947, after numbers in the group had been increased.

Failures in breeding and lactation in zoo populations, as in other groups that have been studied, may be expected to affect the subordinate animals and their offspring most severely. Such responses would contribute to natural selection. Similarly, inadequate development of offspring and their susceptibility to micro-organisms also would contribute to selection. On the other hand, arterial disease

has not as yet been a factor in selection, for it has been a cause of death only of animals that had attained reproductive age.

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*ON BEING JUST COMPLICATED ENOUGH**

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One would suppose that as human cultures have become more complex, in the course of their evolution from paleolithic to modern industrial levels of organization, the various sub-systems—kinship, economic organization, political structure, etc.—of which the original cultures were composed would also have become more complex. In particular, in the realm of social culture, one would expect that as the size of kindreds and bands increased with rising life expectancies, and as new social groups came into existence, a more complex system of kinship terminology would develop to specify the (presumably) increasingly complex varieties of relationships among kinfolk. But we are faced with a paradox. Despite the early expectations of anthropologists that (to quote Service[9])

> "... the tremendous changes in types of society from the simple hunting-gathering bands to the complex modern states which have accompanied the evolution of culture would be reflected in significant and far-reaching alterations in basic patterns of kinship terminology that could be understood in relation to that evolution,"

it has not been possible to demonstrate any such relationship, either historically or in cross-cultural comparison of societies of different evolutionary positions. Indeed, the discouragement of finding no relation at all between evolutionary level and type of kinship terminology has led some to raise the question (rhetorically, at least) of whether there is any important functional connection between kinship terminological systems and other aspects of social structure.

Working along somewhat different lines than the writer, Service has dealt with the paradox in a recent paper on kinship terminology and evolution (Service, 1960). His solution is the observation that, while there is no relation between evolution and type of *kinship* terminology (e.g., between emphasis on generation as a principle of classification and per capita energy control by the society), there is a relationship between evolutionary level and the number of sub-systems of the *status* terminology system (of which kinship terminology is but one sub-system). He classifies status terms as egocentric-familistic (EF) (relational kinship terms), sociocentric-familistic (SF) (kinship group terms, such as clan names), egocentric-

nonfamilistic (EN) (such as class-and-caste or feudal relational terms like "slave," "Lord," etc.), and sociocentric-nonfamilistic (SN) (terms denoting organizational membership or status). With such a classification, it is possible to establish a valid evolutionary partial-ordering:

$$EF \leftarrow SF \leftarrow EN \leftarrow SN$$

This schema does relate types of status terminologies to evolutionary level, and recognizes that the evolution is accompanied by an increase in the number of subsystems—a point to which we shall return. But it does not explain why there has been no change in either the type or the semantic complexity of the kinship (egocentric-familistic) terms themselves as the status terminology system as a whole has become more complex.

The Quantitative Measurement of Semantic Complexity.—Before we can deal effectively with the problem of complexity, it is necessary to develop a quantitative measure. For taxonomic systems reflected in terminology, it is possible to use a measure of semantic complexity analogous to information measure as described by Shannon.[10] This measure of semantic complexity depends upon the use of a technique known as componential analysis, developed in the last few decades by anthropologists and linguists[1, 4, 11] for the purpose of describing objectively the meaning of the terms in taxonomic lexicons, particularly kinship terminologies.

Componential analysis as a method of semantic investigation consists of five steps: (1) the recording of a complete set (or a defined sub-set) of a single taxonomic lexicon, using one or another boundary setting criterion, such as a constant syntactic context, a type of pragmatic situation, or common inclusion under a cover term (such as, for English kinship terms, the term "kinsman"); (2) the definition of these terms by reference to a standard set of denotations or referents (for example, in kin term analysis, the traditional kin-type denotata such as mother's brother, father's sister's husband, etc.); (3) the identification, in the principles of the grouping of denotata, or two or more conceptual dimensions (together constituting a taxonomic space) each of whose values ("components") is signified (not connoted) by one or more of the terms; (4) the definition of each term as a specific combination, or set of combinations, of the components; (5) a statement of the logico-semantic relationships among the terms and of the structural principles and mathematical properties of that dimensional and terminological system. Several interconvertible notational systems, both algebraic and graph-form, can be used to represent a componential analysis. As an example of the product of a componential analysis of a portion of a kinship terminology, see the representation of certain terms of American English in Table 1.

Now, how can one measure the semantic complexity (within the boundaries of a defined lexicon) of a single term? This can be done quite precisely, by counting the number of cells (N_t) occupied by the term, and dividing that number by the number of cells (N_s) in the taxonomic space. The fraction N_t/N_s, or a function thereof, will be a measure of the semantic complexity of the term in question. If one wishes to measure the semantic complexity of the whole taxonomic lexicon, one may calculate the mean semantic complexity of the several terms. This last operation will yield a value for mean N_t/N_s which cannot be larger than $1/L$, where L is the number of terms in the lexicon.

A convenient function of the values N_t/N_s, mean N_t/Ns, and $1/L$, is their negative logarithm to the base 2. These $-\log_2$ functions indicate the minimum number of binary discriminations necessary to the specification of the meaning of the term, and in the case of mean N_t/N_s and $1/L$, indicate the minimum number of binary dimensions necessary for the construction of a taxonomic space on which every term of the lexicon can be specified.

TABLE 1

A COMPONENTIAL PARADIGM OF SELECTED AMERICAN ENGLISH CONSANGUINEAL KINSHIP TERMS*

	LINEAL		CO-LINEAL		ABLINEAL	
	♂	♀	♂	♀	♂	♀
2nd Ascending Generation	GRAND FATHER	GRAND MOTHER	UNCLE	AUNT	COUSIN	
1st Ascending Generation	FATHER	MOTHER				
Ego's Generation	[EGO]		BROTHER	SISTER		
1st Descending Generation	SON	DAUGHTER	NEPHEW	NIECE		
2nd Descending Generation	GRAND SON	GRAND DAUGHTER				

* From Wallace and Atkins, 1960 and Goodenough, private communication.
Lineals are persons who are ancestors or descendants of ego; *co-lineals* are nonlineals, the set of all of whose ancestors includes, or is included in, the set of all the ancestors of ego; *alineals* are consanguineal relatives who are neither lineals nor co-lineals.

Thus we may take $\log_2 L$ $(= -\log_2 1/L)$, where L = the number of mutually exclusive terms in a defined lexicon, as the asymptote of all possible estimates of the semantic complexity of that taxonomic system. This number also indicates the minimum number of binary dimensions necessary to the construction of a taxonomic space adequate to contain all the terms of the lexicon, and, again, its anti-logarithm is the maximum number of entities in the lexicon. From its analogy with the concept of statistical information, which also employs the $\log_2$ function as a measure of the complexity of a system of binary choices, we shall denoté the functions $\log_2 L$, $-\log_2 N_t/N_s$, and $-\log_2$ mean N_t/N_s by the symbol H_{sem}.

The Semantic Complexity of Kinship Terminologies Compared.—Let us consider a group of six well-described kinship terminologies from societies of varying levels of complexity: the Kariera of northwestern Australia,[7, 8] the Comanche of the southern United States (unpublished data supplied by Ward H. Goodenough), the Lapps of northern Sweden,[6] the Trukese of Micronesia,[1] the Japanese (un-

published data collected by the author and John Atkins), and the United States. For each of these societies, a reasonably complete lexicon of kinship terms is available. The number of terms ranges from fourteen, as reported for the Trukese, to forty-nine, for the Lapps. The number of binary dimensions necessary for the semantic specification of these terminological systems ranges from four, for the Trukese, to six, for the Comanche, Lapps, Japanese, and United States (Table 2).

TABLE 2

THE MINIMUM MEAN SEMANTIC INFORMATION QUANTITY (H_{sem}) FOR KINSHIP TERMS IN SIX CULTURES

Society and locality	Number of terms	Minimum H_{sem}	Order of societal population size	Technological level
KARIERA (Australia)	23	4.52	Hundreds	Hunting and gathering
COMANCHE (Southwest U.S.)	37	5.21	Hundreds	Hunting and gathering
KÖNKÄMÄ LAPPS (European arctic)	49	5.64	Hundreds	Nomadic reindeer herding
TRUK (Micronesia)	14	3.82	Thousands	Horticulture and fishing
JAPAN	39	5.29	Millions	Modern industrial
UNITED STATES	37	5.21	Millions	Modern industrial

Two facts are apparent, even when this small group of terminologies is considered: first, there is no *necessary* relation between complexity of the kin terminology system and the size and technological level of the society; and second, each of the systems can be accommodated by a taxonomic space requiring only six binary dimensions or less. While it would be hazardous to claim that there are no kin terminologies amounting to more than sixty-four mutually exclusive terms (the maximum number which can be contained in a space of six binary dimensions), those systems chosen by anthropologists for componential analysis have not approached this number. For the moment, therefore, let us work with the hypothesis that six binary dimensions are sufficient to define any kinship terminological lexicon.

The Size of Taxonomic Spaces in Other Taxonomic Systems.—Do we find the same value, a taxonomic space of six binary dimensions, as a limit on the complexity of other culturally institutionalized systems of discrimination? Let us consider the phonemes of which language is composed. The range in the number of segmental phonemes in natural languages on which a reliable phonemic analysis has been reported is from 13 to 45.[3] Thus all phonemic systems can be plotted on taxonomic spaces of six binary dimensions or less. Alphabets, as distinct from syllabaries and hieroglyphic systems, are crude graphic approximations of phonemic systems, and, presumably, also observe the limit of 2^6 units.

But we can approach the question vertically, in depth within a single society, as well as horizontally, in cross-cultural comparison. Looking at the contemporary culture of the United States one can identify a number of taxonomic systems, in addition to phonemes, and kin terms, which fall within the hypothesized 2^6 limit for number of entities in the lexicon. Thus, for instance, English verb paradigms would, superficially, seem to involve at least seven dimensions, some of them containing more than two values. If these dimensions were independent and thus orthogonally related—that is to say, if each value on each dimension could combine with all possible combinations of values on the other dimensions—the resulting taxonomic space would contain 576 cells, requiring 2^{10} binary dimensions. But as a matter of fact these dimensions are not independent, and in any case the number of grammatically significant forms of an

English verb is less than 64—the system requiring, again, no more than the equivalent of 6 binary dimensions for its semantic explication. The same may be said for a variety of games: the number of squares on a chessboard is exactly 64, and the number of pieces even less; the number of players in field sports is less than 64; there are only 52 distinguishable types in a standard deck of playing cards; there are 12 combinations in a pair of dice; the number of lexemes in our number system is less than 64; the number of types of military rank is less than 64; and so on *ad infinitum*.

It is, however, also possible to find some instances of American taxonomic or discrimination systems which exceed 64 units: for instance, the 88 keys on a modern piano, and the 84 symbols of a standard typewriter keyboard. And there are the various scientific taxonomies, such as the system of biological classification, and the table of the elements, which greatly exceed the 2^6 limit. But those exceptions, and probably others which could be cited, seem to differ from the set of simpler "folk" taxonomies in two major respects: not everyone is expected to "master" them, and they require more or less protracted specialized training for their successful manipulation.

The "Two-to-the-Sixth-Power" Hypothesis and Its Corollaries.—Let us now formulate more precisely, as an hypothesis, some of the foregoing considerations, and provide more explicit definition of certain of the concepts invoked. By a taxonomy we mean a group of symbols (a "lexicon" or "nomenclature") which denote mutually exclusive but jointly exhaustive subsets of referents within a set denoted by a cover symbol and which (unlike names) are defined by unique combinations of values on two or more conceptual dimensions. (Thus all members of a taxonomic lexicon are of the same logical type.) By a folk taxonomy we mean a taxonomy which is used by all normal members of a human society and which does not require specialized training for successful use. The hypothesis, which we shall call the "2^6 Rule," is, then, that *irrespective of race, culture, or evolutionary level, culturally institutionalized folk taxonomies will not contain more than 2^6 entities and consequently will not require more than six orthogonally related binary dimensions for the definitions of all of the terms.*

It would seem that we are confronting a problem similar to that discussed by J. B. S. Haldane[2] in his well-known paper "On Being the Right Size." Haldane, of course, had little to say about the "sizes" of the cultural aspects of human behavior. His point was that, given constant certain anatomical and physiological parameters, the application of some simple mathematics of dimensional analysis would indicate limits of possible physical growth. In the area of cultural semantics, we are suggesting that a somewhat similar principle applies, a corollary of the 2^6 Rule, to wit: *the evolution of cultural complexity is limited, in so far as folk taxonomies are concerned, by the two-to-the-sixth-power rule.* Obviously, however, the limitation imposed on cultural growth by this corollary does not affect either the population of the society, the total or per-capita amount of energy under its control, the number of mechanical devices it can produce, the total number of words in its language, nor the number of separate social institutions which it can maintain. What is limited is the complexity of the taxonomies which are components of the various cultural sub-systems. Thus, as one moves from a "simple" herding culture like that of the Lapps to a "complex" industrial culture like that of the Japanese or

the Americans, one does not find that the increase in cultural complexity has been accompanied by the development of substantially more complex phonemic or kinship taxonomies, or (hypothetically) by more complex folk taxonomies of any kind. Rather, it has been accompanied by (as Service[9] points out) by the addition of new taxonomies, some of them (those exceeding the 2^6 Rule) dependent for their use on training of selected recruits in the use of specialized literary and technological skills.

Consideration of the quantitative limits of folk taxonomies may also remind the reader of Miller's 1956 paper[5] bearing the fascinating title, "The magical number seven, plus or minus two: Some limits on our capacity for processing information." Miller, in this review (which Dr. L. S. Rubin drew to my attention after I had drafted the present paper) of psychological experiments dealing with the information channel capacity of human beings, found that "absolute judgments of unidimensional stimuli" were accurate only for seven or fewer stimuli (i.e., the H_{sem} values were less than three). For multidimensional stimuli, the H_{sem} values implied by limits of efficient discrimination increased to between two and seven, depending on the problem, with 7.2 the maximum obtained figure (for acoustical variables). It would appear, then, that our 2^6 Rule is not far from describing the results obtained in the psychological laboratory when discriminations among stimuli characterized by different values on two or more dimensions are the task at hand.

Implications of a Taxonomic Complexity Rule for the Evolution of Man and of Culture.—It is evident that the numerical value of 2^6 for maximum size of folk taxonomies is a crude estimate based on a very small group of observations. The number 2^6 may prove to be too small (it certainly is not too large). The precision of the 2^6 Rule is not, however, as important as the propositions that, first, a 2^6 Rule can be formulated on the basis of fuller empirical evidence, and, second, that such a rule reflects a cognitive limit, constant for human populations, on the complexity of semantic spaces with which all but a few individuals in any society can reliably and comfortably function.

Such a rule would have, I believe, some utility as an explanatory device in considering the relations between physical and cultural evolution.

Let us approach these relations in the light of the fact that both man's physical varieties and his cultural varieties are subject to different selective processes in the course of their respective evolutions. These selective processes can, in a sense, work at cross purposes. Thus, as it has often been remarked, societies do not always select "efficiently" for physical (including neurological) characteristics. Societies sometimes "make do" with what may be, in a biological sense, inferior members because at any point in time the cost of eliminating them, however "desirable" this might appear to be from an abstractly eugenic standpoint, would be higher than the society itself could afford to pay. In other words, social survival may require a partial suspension of "natural" selection. Hence, in general, in the course of the evolution of any human population, there must occur a sort of compromise between the pressures of natural selection and the pressures of cultural selection.

Now the content of this compromise evidently will have a bearing both on physical and cultural evolution. And a taxonomic complexity rule will play a vital part in

defining that content. Specifically, it will function, with respect to physical evolution, as a pruning hook to eliminate from full reproductive participation those individuals unable to handle, regularly and efficiently, taxonomic systems approaching 2^6 in complexity. And it will also function, with respect to cultural evolution, as a pruning hook to preclude, or, if introduced, to eliminate those cultural innovations which would require of populations generally the manipulation of excessively complex taxonomic systems. The visible effects of this compromise, finally, ought to be evident in two phenomena: first, the definitions of mental incompetency and the methods for handling mental incompetents; and, second, the dependence of societies, as their cultures increase in complexity, on adding new folk taxonomies (rather than on making old ones more complex) and on training selected persons (or, today, machines) in the use of technologically necessary taxonomies which exceed the 2^6 Rule. Such a process—to return at last to the problem of crowding and stress which inspired this symposium—should have the effect of protecting the human organism from what may be called psychological crowding. As the numbers of persons in a society increases, as the population density rises, as the technology becomes more complex, the individual nonetheless is required to use no more complex taxonomies than he had in past times. Thus, *psychologically* speaking, it seems likely that the primitive hunter and the urban technician live in cognitive worlds of approximately equal complexity and crowdedness.

* Presented at the symposium under the title "The Complexity of Cultures."

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INTERACTION OF SMALL IONS AND MOLECULES WITH RIBONUCLEIC ACID

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A systematic study of the effect of metallic ions and salts upon the sedimentation properties of ribonucleic acid (RNA) was undertaken in order to better understand the role of these substances in the reversal of the dissociation of RNA by proteins previously described.[1] The results were unexpected in that certain concentrations of salts alone produced a reversible decrease in the sedimentation rate of RNA. This paper is a preliminary report of the effect of certain salts and small molecules upon RNA sedimentation and a discussion of the implications of these data.

The high-molecular-weight RNA used in the experiments was isolated by phenol extraction[3] both from Ehrlich ascites tumor cells and from the L-5178Y tissue culture line of mouse ascites cells.[2] The high-molecular-weight fraction from the Ehrlich ascites RNA was separated by 1 *M* NaCl precipitation, and that from the L-5178Y cells by streptomycin precipitation.[4] Results with both types of RNA were similar and no distinction will be made between them.

All ultracentrifugal analyses were performed in the Spinco model E ultracentrifuge using ultraviolet absorption and the Analytrol with the microanalyzer attachment.[5] Unless otherwise stated, the RNA concentration was 25–30 γ/ml. With the exception of the experiments in which the effect of nonalkali metals were being studied, all solutions contained 0.001 *M* versene. Quantitative measurements of ultraviolet absorption were made in the Beckman DU spectrophotometer.

Edelman *et al.*[6] have shown that one equivalent of Mg^{++} can be bound to each nucleotide in RNA. The apparent density of 2.03 for RNA in concentrated CsCl observed by Davern *et al.*[7] can best be rationalized by assuming that one Cs^{+} per nucleotide is bound to the RNA. A reasonable assumption would be that the binding of metallic ions inhibits, or reverses, the dissociation of RNA by proteins. Supporting this hypothesis is the observation that an increase in the sedimentation rate for RNA is observed at the same molarity effective for the inhibition of the dissociation of RNA by proteins. The increase in sedimentation rate appears to be proportional to the density of the metallic ion, i.e. RNA sediments more rapidly in potassium chloride solutions than in sodium chloride solutions. Inhibition has been observed for the following ions at the indicated molarities: Na^{+} −0.6 *M*; K^{+} −0.6 *M*; Mg^{++} −0.005 *M*; Ca^{++} 0.01 *M*; Ni^{++} −0.005 *M*; Mn^{++} −0.0005 *M*. All solutions were buffered at pH 7.4 with 0.01 *M* tris hydroxy amino methane (tris). The protein used was γ-globulin at 50 γ/ml and the sodium and potassium were added as the chlorides. The other ions were in 0.14 *M* NaCl solutions.

A more complicated phenomenon was observed when RNA alone was sedimented in NaCl and KCl solutions at molarities between 0.20 and 0.30 *M*. At these molarities the boundaries became blurred and the sedimentation rates decreased. The reaction was a slow one, taking approximately 6 hours to reach an equilibrium at 4°C. In NaCl solutions, the largest decrease in sedimentation rate was observed to take place at 0.24 *M*. The optimum molarity for the decrease in KCl was 0.30 *M*.

Sedimentation rates were slower in 0.30 *M* KCl than in 0.24 *M* NaCl. Not only was there an optimum molarity, but there was also an optimum pH: in solutions buffered with 0.01 *M* phosphate, the largest decrease took place at pH 6.9–7.2 and the same pH optimum was observed in tris-buffered 0.30 *M* KCl. The decrease in sedimentation rates was dependent upon the concentration. The RNA in a solution containing 1,500 γ/ml of RNA sedimented more rapidly than did the RNA in a solution containing 30 γ/ml.

The reaction which takes place in 0.30 *M* KCl does not result in any increase in the ultraviolet absorption. Equal concentrations of RNA in 0.14 *M* NaCl and in 0.30 *M* KCl solutions, both buffered at pH 6.9 with 0.01 *M* phosphate, were incubated for 6 hours at 4°C and the ultraviolet spectra measured. The results are presented in Figure 1.

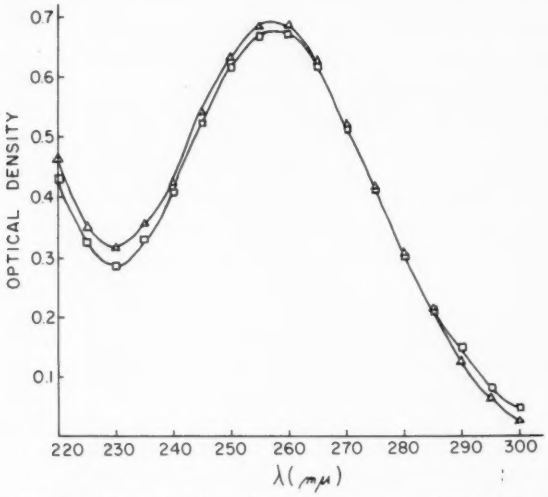

FIG. 1.—Ultraviolet absorption of equal concentrations of RNA in: ⊡ 0.14 *M* NaCl and △ 0.30 *M* KCl at pH 6.9 after incubation for 6 hours at 4°C.

Both the cation and the anion are important in the interaction of salts with RNA. There was no decrease in the sedimentation rates at any molarity of LiCl or $NaClO_4$. There was not, however, complete specificity. Sedimentation rates decreased in all of the potassium halides. In tris solutions the decrease occurred between 0.20 and 0.30 *M* with an optimum molarity of 0.30 *M*. The pH of the tris was adjusted with concentrated hydrochloric acid and the anion present was the chloride. The decrease in sedimentation rates became larger with decreasing pH; the decrease in tris was not as great as that observed in 0.30 *M* KCl at pH 6.9.

Uncharged molecules may also interact with RNA and a study of these interactions is being pursued by Dr. Francis Ashton of these laboratories. 0.02 *M* N-methyl urethane (ethyl methyl carbamate) in 0.14 *M* NaCl has been found to be an optimum concentration for producing a decrease in the sedimentation rates of RNA. In contrast to 0.30 *M* KCl, the decrease in the sedimentation rates observed

with N-methyl urethane was not dependent upon pH. The absence of pH dependence should probably be correlated with the lack of a charge on the N-methyl urethane molecule.

Sedimentation analysis can be used to study the interaction of various molecules with RNA. Experiments in column chromatography and stability studies have suggested that amino acids do interact with RNA. Amino acids affect the sedimentation rate in a rather complex fashion and a study of these interactions will be the subject of a future communication. Figures 2*b* and 2*c* compare the ap-

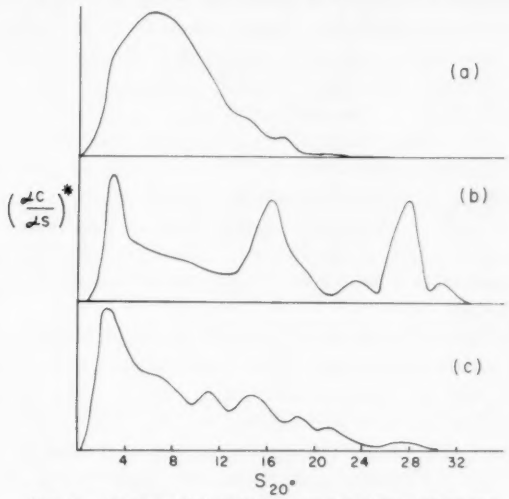

FIG. 2.—Apparent (8) distributions of sedimentation constants of RNA in: (*a*) 0.30 *M* KCl buffers at pH 6.9; (*b*) 0.20 *M* tris pH 7.4 containing L amino acids; (*c*) 0.20 *M* tris pH 7.4 containing D amino acids.

parent[8] distributions of sedimentation constants obtained in a 0.20 *M* tris solution pH 7.4 containing equivalent mixtures of L and D amino acids (alanine, asparagine, aspartic glutamic, histidine, isoleucine, leucine, methionine, serine, threonine, valine) all at 30 γ/ml and phenylalanine, tryptophan at 20 γ/ml. The large differences in the sedimentation distributions observed in equal mixtures of D and L amino acids demonstrate clearly that there are specific interactions between amino acids and RNA. In the D amino acids a major portion of the RNA sediments at a rate of 3 S (Svedberg's).

The decrease in sedimentation rates of RNA in 0.30 *M* KCl and 0.30 *M* tris was reversed by adding 0.005 *M* Ni^{++} or 0.005 *M* Mg^{++} and allowing the solution to stand overnight at 4°C. There were, however, differences between this material and the control. In Figure 2*a* is shown the apparent distribution of sedimentation constants observed for high molecular weight RNA after 6 hours at 4°C in 0.30 *M* KCl buffered at pH 6.9. At that time, sufficient solid KCl was added to make the solution 0.80 *M* KCl. The material was analyzed in the ultracentrifuge after it had been allowed to stand overnight at 4°C and compared with material which had originally been diluted in 0.80 *M* KCl. The two patterns were identical, even in

their fine detail. By the criterion of sedimentation analysis, the reaction is completely reversible. However, the clean recovery of the original sedimenting components was only possible with the use of undegraded RNA. Reversal experiments using RNA which had been stored for six months at −70°C were not completely successful.

The observed decrease in sedimentation rates could arise through the dissociation of the molecule, a change in shape of the molecule, or a combination of both. The dependence upon concentration of the sedimentation rates provides strong evidence for dissociation. Doty *et al.*[9] have shown that the transition[10] of the RNA molecule to a more extended form in solutions of low ionic strength is accompanied by a substantial increase in the optical density at 260 mμ. Any degradative procedure when applied to RNA also leads to an increase in the optical density.[11] The decrease in the sedimentation rates under discussion was accompanied by a very small change in the ultraviolet absorption, and it is unlikely that there was either degradation of the RNA molecule or a transformation to a more extended form. This evidence also favors dissociation. In solutions of low ionic strength, the sedimentation boundaries observed with high molecular weight RNA move more slowly but remain distinct. The broad distribution of sedimentation constants observed in 0.30 *M* KCl is suggestive of dissociation rather than of an increase in asymmetry.

In the D amino acids, a substantial portion of the high-molecular-weight RNA had a sedimentation constant of 3 S. This value is incompatible with the molecular weight found for high-molecular-weight cellular RNA by means of light scattering.[12] Soluble RNA has a sedimentation constant of 4.0–4.2 S and contains 70–80 nucleotides.[13] It would be reasonable to assume that an RNA molecule with a sedimentation rate of 3 S contains 40 nucleotides, although the number may be as large as 100.

It appears that all of the high-molecular-weight RNA can be dissociated into small pieces containing 40–50 nucleotides, and it is convenient to call these pieces subunits although they cannot be identical. The ultraviolet absorption data prove that these subunits are not held together by hydrogen bonds of the type found in the DNA double helix.[14] The importance of both cations and anions in the dissociation suggests that an ionic bond is involved. Since there was no pH dependence of the dissociation by an uncharged molecule, N-methyl urethane, the most reasonable explanation for the pH optimum in 0.30 *M* KCl is that the two ions can best compete for the two complementary sites on the RNA subunits when the sites carry a charge. The pH optimum suggest two ionizable groups, one with a pK above and the other with a pK below 7.

RNA has been shown to interact specifically with amino acids. Using similar techniques, it may be possible to show specific interactions with other biologically important molecules.

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*THE DE NOVO SYNTHESIS OF POLYSACCHARIDE BY PHOSPHORYLASE**

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The phosphorylase reaction can be represented by the following equation:

$$\alpha\text{-glucose-1-phosphate} + [G]_n \rightleftharpoons [G]_{n+1} + P_{\text{inorganic}} \quad (1)$$

$[G]_n$ stands for a "primer" which can accept glucose residues from G-1-P in the elongation of chains. When $[G]_n$ was omitted, that is, when highly purified muscle phosphorylase was incubated for 20 minutes with G-1-P, there was no exchange of P_i^{32} with G-1-P under conditions which resulted in a substantial reaction in the presence of $[G]_n$.[1, 2] There was also no detectable liberation of P_i during such an incubation when $[G]_n$ was omitted. Similar results were obtained with potato phosphorylase.[2] The interpretation of these observations has been that phosphorylase cannot start a polysaccharide chain.

In earlier experiments, when no polysaccharide primer was added to incubation mixtures containing partially purified phosphorylase, P_i liberation was eventually observed after lag periods of various durations. These lag periods could be shortened or abolished by the addition of minute amounts of glycogen.[3] Furthermore, the addition of extracts containing "branching enzyme" made the system seemingly self-starting in the absence of added primer, and, under some conditions, the liberation of P_i followed an autocatalytic course.[4] This effect could be ascribed to the creation of more and more end groups by successive branching of lengthened linear chains. In none of these cases could proof be offered that the enzyme and G-1-P preparations used were free of traces of primer. How difficult a task this is will be shown below.

Recently Lee, in this laboratory, purified potato phosphorylase by electrophoretic separation on cellulose powder and obtained a preparation which was

homogeneous in the ultracentrifuge and homogeneous on electrophoresis at pH 7.5, but not at pH 6.9 or 7.2.[5] This enzyme preparation, in amounts much greater than were required in an ordinary catalytic system, liberated inorganic phosphate at a slow rate after a lag period of several hours, even when synthetic G-1-P was used instead of enzymatically prepared G-1-P which is often contaminated by activating polysaccharides. The synthesis of new polysaccharide in the "primer-free" system was demonstrated by the appearance of material staining blue with iodine. Reports have appeared in the literature that plant phosphorylases from other sources do not require a "primer" for activity.[6-8]

These observations have prompted a reinvestigation of the question of the ability of phosphorylase to catalyze the *de novo* synthesis of an amylose-like chain. In the experiments to be discussed it will be shown that muscle phosphorylase preparations can be obtained which meet rigid criteria of being virtually free of carbohydrate and of "branching enzyme." Such preparations are still capable of *de novo* synthesis, that is, they can initiate the formation of a polysaccharide chain from G-1-P. The initial act, the *de novo* reaction proper, occurs at an exceedingly slow rate and is characterized by an apparently absolute lag period during which no P_i is liberated and no exchange occurs with P_i^{32}. This is followed by the more rapid reaction of chain elongation which becomes freely reversible when 4 to 5 glucose units have been added and which can lead to the formation of chains more than 2,000 units long. Early intermediates of chain growth consisting of 2 to 6 glucose units attached to the enzyme have been demonstrated. In the absence of all traces of contaminating α-amylase and of exogenous primer, only a small number of amylose chains are formed per enzyme monomer because of the low rate of the *de novo* act. In the presence of highly purified "branching enzyme" and phosphorylase the rapid formation of glycogen can be initiated by the *de novo* reaction. Various features of these reactions will be discussed.

Experimental.—Crystalline phosphorylase *a* was prepared as described by Cori, Illingworth, and Keller,[9] and was recrystallized from EDTA-glycerophosphate buffer from 4 to 10 times. Exhaustive recrystallization is known to be necessary to free phosphorylase from traces of amylo-1,6-glucosidase activity, and was, therefore, also desirable in this study where an enzyme of the greatest purity was required. In addition to repeated recrystallization, the purification of phosphorylase *a* on DEAE cellulose columns was studied, particularly from the point of view of freeing it from any traces of α-amylase and of the amylo-1,4 → 1,6-transglucosidase ("branching enzyme").

Column chromatography was done at 4° using DEAE cellulose which had been treated with NaOH and then washed exhaustively with water. The column (1 × 18 cm) was washed overnight with about 1,500 ml of 0.005 *M* Tris–0.005 *M* mercaptoethanol-0.001 *M* EDTA, pH 7.2 to 7.4. Crystals of phosphorylase *a* were dissolved in a small volume and the solution dialyzed overnight against a large volume of the buffer. The column was loaded under gravity flow, and a salt gradient established by using a reservoir of the buffer containing 0.3 *M* NaCl and a mixing vessel containing 375 ml of the buffer alone. The column was operated at a flow rate of about 16 ml per hour. Fractions were collected automatically at 15-min intervals.

It was found that salivary and pancreatic α-amylases have a very weak affinity for such a DEAE cellulose column. Amylase activity, therefore, can be slowly washed through with buffer containing no NaCl, and is very rapidly eluted as the salt concentration increases following establishment of the gradient. However, if any substantial amount of amylase is present in a solution added to the column, traces of activity corresponding to 0.01 to 0.5 μg per ml of the enzyme can still be detected even after 200 ml of eluent have passed. If the "branching enzyme" is present, it is eluted early in the gradient, often after only 30 ml of eluate have been collected. Phosphorylase *b*, if present, is not well resolved from the "branching enzyme." Amylo-1,6-

glucosidase is eluted as a discrete peak coming near but following that of the "branching enzyme." Traces of activity due to amylo-1,6-glucosidase persist into the phosphorylase *a* peak which follows it. For this reason, phosphorylase *a* was recrystallized until free of the glucosidase before subjecting it to column chromatography when an enzyme of ultimate purity was desired. When 15 mg or more of phosphorylase *a* were put on a column, the enzyme crystallized spontaneously from the peak eluate tubes containing it. These crystals had the same specific activity in every tube in which they occurred. The activity of the enzyme was very high—between 2,500 and 3,200 units per milligram. It was stable over a long period of time when kept in the cold as a crystal suspension in the tris-mercaptoethanol-EDTA buffer, pH 7.3, containing from 0.1 *M* to 0.2 *M* NaCl.

Results.—Reaction without branching enzyme: Figure 1 shows the time course of liberation of inorganic phosphate when 1.06 mg per ml of phosphorylase *a* (sp.

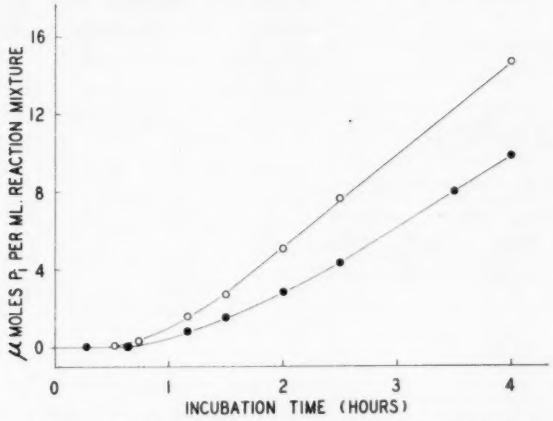

FIG. 1.—The *de novo* reaction catalyzed by phosphorylase *a*. Comparison of chemically synthesized G-1-P (○) and G-1-P prepared from starch (●). Phosphorylase *a* eluted from DEAE cellulose column and recrystallized from EDTA-mercaptoethanol-glycerophosphate, 1.06 mg/ml; 0.04 *M* G-1-P; 0.001 *M* 5′-AMP; pH 6.7; 30°.

act. 3,000 units/mg) was incubated at 30° with G-1-P and 5′-AMP at pH 6.7. The behavior of the system when synthetic G-1-P was used is compared with that when a seemingly pure, commercial sample (Schwarz, Lot No. 5102) was used.[10] A significant feature of the reaction is that no detectable P_i is formed until after a long lag period. Figure 2 shows that the amount of enzyme affects the length of the lag period and that the final rate of the reaction is proportional to the enzyme concentration. At the low levels of enzyme used in conventional phosphorylase assays (*ca.* 0.01 mg per ml), the lag period may be as long as 24 hours or more and hence, was undetected in earlier work. However, even when the enzyme concentration is very high (5 mg per ml), there is still an absolute lag period during which no liberation of P_i can be detected with a method sensitive to a change of 0.04 μmole P_i per ml of reaction mixture. In a particular experiment P_i was found to the extent of about 1 mole per mole of monomer of enzyme (mol. wt. 125,000) at 1, 25, 50, and 80 minutes. Thus, *de novo* synthesis did not occur at all until after 80 min of incubation.

During the course of the reaction described in Figures 1 and 2 a polysaccharide

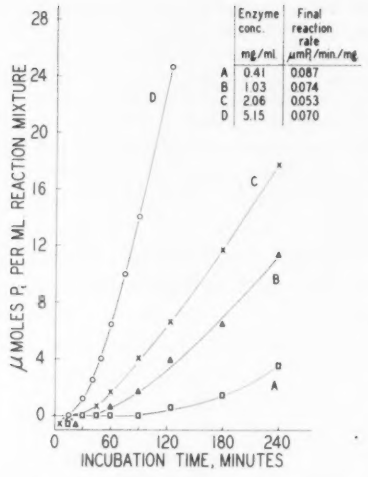

FIG. 2.—Effect of enzyme concentration. Crystalline phosphorylase *a* eluted from DEAE cellulose column; concentrated by centrifugation; 0.08 *M* G-1-P (Schwarz); 0.001 *M* 5′-AMP; Tris-mercaptoethanol-EDTA; pH 6.7; 30°.

is produced which gives an intense blue color upon the addition of iodine. Enzymatic and physical characterization showed that it consists of straight chains like amylose. These chains precipitate early during the reaction period and may dissolve again at later stages, as the phosphorylase equilibrium between G-1-P and P_i is approached.

Absence of primer in de novo system: The final rate of the reaction in Figures 1 and 2 is only a small fraction (about 0.1 per cent) of the rate observed with glycogen as acceptor in a conventional assay. How sensitive the *de novo* system is to trace amounts of glycogen is indicated by the fact that the addition of 3 μg per ml, corresponding to $1.3 \times 10^{-6} M$ end group, abolishes the lag period (Fig. 3*a*). That this effect is due to the priming action of glycogen is shown by subtracting the amount of P_i formed in a system with no glycogen from that with added glycogen. The resulting curves show that the outer chains of glycogen are

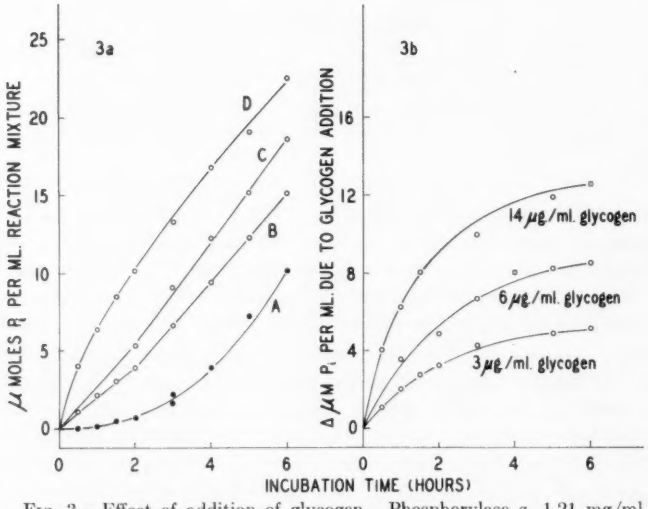

FIG. 3.—Effect of addition of glycogen. Phosphorylase *a*, 1.21 mg/ml, corresponding to $1 \times 10^{-5} M$ monomer; no 5′-AMP; 0.046 *M* G-1-P (Schwarz); pH 6.7; 30°. A, *de novo* control; B, 3 μg/ml glycogen added; C, 6 μg/ml glycogen; D, 14 μg/ml glycogen. In Fig. 3*b* the P_i formed by the *de novo* reaction has been subtracted for each time point and the resulting data plotted.

being elongated and are responsible for an amount of G-1-P reaction proportional to their concentration (Fig. 3*b*). It will now be appreciated that before the existence of a *de novo* reaction could be established with certainty, it was necessary to exclude the presence of trace amounts of primer in the enzyme preparation and in the G-1-P solution used.

Direct analysis by microenzymatic methods of phosphorylase *a* crystals following heating at 100° in 1*N* HCl for 3 to 4 hours usually shows that traces of glucose are present. This carbohydrate content falls to a very low value with repeated recrystallization of the enzyme. Ten milligrams of phosphorylase *a* which had been recrystallized three times were heated in 1*N* HCl for 3 hours. There was no detectable glucose by microenzymatic assay. Ten milligrams of this enzyme were also incubated at 37° with 6 mg of crystalline pancreatic α-amylase for 90 minutes. After deproteinization with 1*N* $HClO_4$ followed by hydrolysis in this acid for 3 hours at 100°, microenzymatic assay revealed the presence of 0.023 μmole glucose or less than 0.3 glucose residue per monomeric unit (mol. wt. 125,000).

On the assumption that there might be some significance to this less than stoichiometric amount of polysaccharide glucose, 80 mg of recrystallized phosphorylase *a* were incubated with 4.8 mg of crystalline salivary α-amylase at 30° for $2^1/_4$ hours. The mixture was then subjected to chromatography on DEAE cellulose under conditions where most of the α-amylase passed into the first column washings. Elution with a NaCl gradient allowed the isolation of crystalline phosphorylase *a*. The peak tube contained 800 μg per ml of this enzyme and about 0.5 μg per ml of α-amylase.[11] When 280 μg of phosphorylase *a* (and about 0.15 μg of α-amylase as contaminant) were incubated at 30° with 0.08*M* G-1-P (commercial or synthetic) and 0.001 *M* 5′-AMP, a slow liberation of P_i began after a long lag period (3 to 4 hours). After 28 hours of incubation, most of the 1-ester had been split and polysaccharide products had been formed which had no I_2 color and which had an average chain length (by reducing power) of 2.7 glucose units. The slowness of the reaction is explained by the fact that the amylose chains formed by phosphorylase were degraded by α-amylase to a mixture of maltose, amylotriaose, and free glucose, none of which has a priming action. These observations show that the *de novo* polysaccharide synthesis does not depend in any way upon the presence in either enzyme or G-1-P of any pre-existing carbohydrate susceptible to α-amylase degradation.

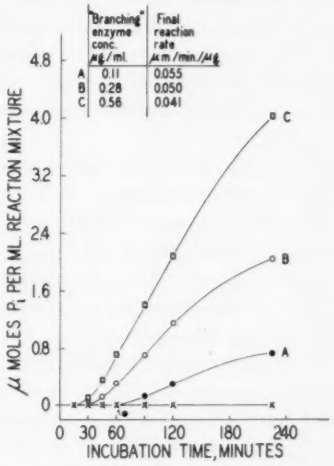

FIG. 4. — Effect of addition of "branching enzyme." Phosphorylase *a*, 13 μg/ml; 0.025 *M* G-1-P (Schwarz); 6×10^{-4} *M* 5′-AMP; Tris-mercaptoethanol-EDTA; 0.15 *M* NaCl (from column buffer and neutralization); pH 6.7; 30°, (×) control without "branching enzyme." "Branching enzyme" addition as indicated.

Effect of addition of "branching enzyme" to de novo system: The availability of highly purified column fractions of amylo-1,4 → 1,6 transglucosidase made it possible to find out whether the *de novo* product can be branched. If this were possible, the addition of "branching

enzyme" should increase the rate of formation of P_i. Figure 4 shows the effect of the addition of three different amounts of "branching enzyme" (0.1 to 0.5 μg per ml) to a catalytic amount of phosphorylase *a* (13 μg per ml) which, by itself, gives no detectable synthesis of amylose. It is seen that the lag period of the reaction is shortened but not abolished, and that the running rate of the reaction becomes enormously greater. In this system both the length of the apparent lag period and the eventual running rate of the reaction are nearly proportional to the concentration of the "branching enzyme." Phosphorylase and this enzyme together constitute a catalytic system, acting rapidly to form a product which has the structure of glycogen (see below). It should be emphasized that the *de novo* reaction is not dependent on the presence of "branching enzyme." The latter merely serves to magnify the effect of the phosphorylase activity by creating multiple end groups.

An interesting feature of the branching reaction shown in Figure 4 is the fact that it proceeds with considerable speed at a very low concentration of the linear chain substrate. Thus, 13 μg of phosphorylase per ml would provide a concentration of chains only 10^{-7} *M* (one chain per monomer) on which 0.1 μg per ml of "branching enzyme" has demonstrable action. Such a system could be of physiological significance and could explain how glycogen synthesis gets started in embryonal tissues. Further purification of the "branching enzyme" and the reaction it catalyzes are under study and will be reported elsewhere.

Product of de novo reaction without "branching enzyme": Phosphorylase was prepared by column chromatography of a 4 times recrystallized enzyme. Every column fraction in the region in which the "branching enzyme" is known to be eluted, was examined for the presence of this enzyme by its catalytic effect on the *de novo* synthesis of polysaccharide by phosphorylase as discussed above. No trace of "branching" activity could be found, and hence, the phosphorylase *a* peak which was eluted much later from the column was considered free of such activity.

The composition of one reaction mixture used for the large-scale preparation of the *de novo* product was: G-1-P, 0.20 *M*; phosphorylase *a*, 710 μg per ml (2,820 units per mg), from DEAE cellulose column; Tris buffer, 0.0025 *M*; mercaptoethanol, 0.0025 *M*; EDTA, 5×10^{-4} *M*; NaCl + KCl, about 0.2 *M*; pH 6.7; volume, 10 ml; 30°. In addition, a second reaction mixture was prepared, in every way identical with the first, except for the addition of 5′-AMP (0.001 *M*). Incubation was under toluene vapor for 30 hours; samples were removed at intervals for inorganic phosphate analysis. At the end of the incubation period the solutions were still clear; in the one with no 5′-AMP, 37 per cent of the G-1-P had been converted to a polysaccharide product (13.4 mg per ml), while 57 per cent had reacted in the mixture containing 5′-AMP to give 20.6 mg per ml of polysaccharide. Each solution gave an intense blue color and a granular blue precipitate when tested with iodine.

An aliquot of two ml of each reaction mixture was incubated with 1.5 mg of pancreatic α-amylase (Worthington, 2 × crystalline) for 3 days at 30° under toluene vapor. An iodine test at that time showed that no chromogenic polysaccharide chains remained. The digests were heated in boiling water and deionized by passage through 1 × 15 cm Amberlite MB-3 columns. After washing

the columns with water, the eluates were evaporated to dryness, taken up in 1.5 ml of water, and one-fifth of the total solution of each (corresponding to 5.3 and 8.2 mg polysaccharide glucose) was spotted on Whatman No. 1 paper for descending chromatography. The papers were developed with butanol/pyridine/water (3:2:1:5) for 74 hours. The position of reducing sugars was revealed by a benzidine-trichloroacetic acid spray. Among the reference standards spotted simultaneously on the paper was a deionized and similarly treated α-amylase digest of 0.5 mg of the branched fraction (amylopectin) from corn starch. This reference digest showed five well-separated, slow moving spots corresponding to various oligosaccharides containing the 1,6-branch points. Maltose was an additional prominent spot. The amylase digests of the polysaccharides formed by phosphorylase action, contained no detectable traces of branched oligosaccharides. Amylotriaose and maltose were the only substances present. Since a digest containing 10 to 15 times as much polysaccharide glucose was spotted in the case of the product synthesized by phosphorylase, it is apparent that these products are linear, i.e., they could contain at most 0.1 per cent branch points.

Determination of molecular weight: In order to characterize further the product of *de novo* synthesis, aliquots of each of the two reaction mixtures (with and without 5′-AMP) described above, were dialyzed for three days against a large volume of distilled water to remove diffusible ions. The dialysis bags then contained a considerable precipitate which was insoluble in hot water. The precipitates were easily soluble in 0.75 *N* NaOH in the cold. One volume of ethanol was added to the cold, alkaline solution and the polysaccharide precipitates removed by centrifugation. The products then dissolved easily in water and were reprecipitated twice with ethanol. They were washed with alcohol and ether and dried. The recovery of polysaccharide by this procedure was complete.

The molecular weights of these products were determined by a viscometric method, based on the use of the modified Staudinger equation

$$[\eta] = \mathrm{K}\cdot\overline{\mathrm{M.W.}}^{\alpha} \qquad (2)$$

The solvent selected for this work was 0.49 *N* NaOH in which all of the polysaccharide products were soluble. The values of the constants K and α, for amylose chains dissolved in such a solution were determined experimentally from intrinsic viscosity measurements, using Cannon-Ubbelohde semi-micro, dilution viscometers and reference amylose preparations of known molecular weight.[12] The latter substances were prepared enzymatically with phosphorylase *a* by adding various numbers of glucose units from G-1-P to amyloheptaose chains. The greatest chain length that could be obtained in this way was 335, under conditions where *de novo* synthesis of amylose chains by the enzyme remained an insignificant part of the total polysaccharide synthesis.

The relative viscosity of each polysaccharide was measured at 25.00° at each of three or four dilutions. Flow times were from 5 to 7 minutes. The accuracy of the over-all dilution in the viscometer and the stability of the polysaccharide in the alkaline solvent were checked by determining its concentration in the initial solution as well as in the final dilution. This was done by hydrolyzing the polysaccharide for 3 to 4 hours at 100° in 1 *N* HCl and then determining the glucose content of the hydrolysate microenzymatically *via* hexokinase and glucose-6-

phosphate dehydrogenase. The viscosity data were treated in two ways. The intrinsic viscosity was estimated from the slope of the straight line plot of specific viscosity *vs.* polysaccharide concentration. It was also calculated as the intercept on the ordinate of the straight line plot of the reduced viscosity *vs.* polysaccharide concentration. As expected, these two calculations were in good agreement, and the average of them is given in Table 1 as the intrinsic viscosity of each substance. The logarithm of the intrinsic viscosity was plotted against the logarithm of the molecular weight for each reference amylose standard. A straight line was fitted to these data by the method of least squares. The equation of this line was found to be:

$$[\eta] = 0.0107 \overline{\text{M.W.}}^{0.768} \quad (3)$$

Using this equation the average molecular weight of the polysaccharide chains produced *de novo* was calculated from their intrinsic viscosity.

The chain length of the products described above in the absence and presence of 5′-AMP, was 1,050 and 1,377, respectively (see Table 1). The maximum absorption of the I_2 complexes of these amyloses was at 642 mμ in both cases. As expected, this wavelength is much longer than that observed (615 mμ) for a chain 335 glucose units long. In the case of another preparation, an amylose with a chain length of 2,520 (ϵ_{max} for I_2 complex: 650 mμ) was formed. These three products were isolated after overnight incubation. Products 1 and 2, Table 1 were isolated after 3 and $6^1/_2$ hours of incubation and shown to have chain lengths falling within the same range.

Absence of terminal phosphate on de novo amylose chain: It was of interest to determine whether the *de novo* amylose chain terminates at what otherwise would be its reducing end in a G-1-P unit. This would be the case if the initial *de novo* act were the transfer of a glucosyl residue to the C_4 position of G-1-P. This question was examined by allowing the synthesis of amylose to proceed in a reaction mixture containing 0.1 *M* G-1-P^{32} (1850 counts/min/μmole),[13] 0.001 *M* 5′-AMP, and 1 mg of phosphorylase *a* per ml. After 23 hours of incubation at 30° the polysaccharide was isolated by dialysis followed by one ethanol precipitation at about pH 4, and three ethanol precipitations at neutral pH. The recovery of the polysaccharide was 91 per cent. One-fifth of the total amount was plated and counted for P^{32}. If the chain length of the amylose synthesized were 2,000, the amount plated would have had 14 cpm above background, if the chains had one P per mole. The shorter the chain length the greater would be the expected radioactivity in the sample. The background of the counter was 17.0 cpm (1,280 counts). The sample planchet had 16.9 cpm. Thus, the amylose synthesized is free of any phosphate group. This finding proves that in the initial *de novo* act P_i is split out. The following paper contains some experimental evidence about the nature of this reaction.

Product of de novo reaction with "branching enzyme": The composition of a reaction mixture for the preparation of the polysaccharide produced by the combined action of amylo-1,4 → 1,6-transglucosidase and phosphorylase was as follows: G-1-P, 0.15 *M;* Tris, 0.003 *M;* mercaptoethanol, 0.003 *M;* EDTA, 0.0006 *M;* NaCl + KCl, about 0.15 *M;* "branching enzyme," 27 μg per ml; phosphorylase *a*, 27 μg per ml (10 times recrystallized, then eluted from DEAE cellulose column);

0.80 ml; pH 6.7, 30°. After 19 hours of incubation, the reaction had reached equilibrium—that is, 79 per cent of the G-1-P had been converted to P_i and a glucose polymer (theory: 17.6 mg of a monohydrate). The product was precipitated with 1.25 volumes of ethanol. It was dissolved and reprecipitated once at about pH 4 and then once at neutrality. 15.6 mg were recovered. The absorption spectrum of the I_2 complex of this material had a peak at 495 mμ. That this polysaccharide had the structure of glycogen was shown by end group determination. After 3 hours of treatment with phosphorylase *a* + amylo-1,6-glucosidase in the presence of P_i, 92 per cent of the polysaccharide was degraded to a mixture of G-1-P and glucose (microenzymatic determination). From the quantity of glucose formed the degree of branching of the original polysaccharide was calculated to be 7.5 per cent. This is within the range found for normal liver and muscle glycogen. The synthetic glycogen was examined at a concentration of 1 per cent in water in the analytical ultracentrifuge. Figure 5 shows its sedimentation pattern; the sedimentation constant is 49.8. Assuming $f/f_0 = 1.65$ and $\bar{V} = 0.65$, the molecular weight is 2,240,000. The glycogen synthesized *de novo* in this experiment is similar in all ways to many normal muscle glycogen samples.[14]

In another experiment a polysaccharide product was prepared by incubating 13 μg per ml phosphorylase *a* and 38 μg per ml "branching enzyme" with 0.18 *M* G-1-P for 6½ hours at 30°. During the later stages of the *de novo* reaction, the rate of P_i formation was equal to that which would occur in a system saturated with added glycogen. The product of the reaction (glycogen) was recovered to the extent of 90 per cent by ethanol precipitation following digestion in NaOH. The glycogen had an end group of 6.3 per cent and an outer chain length of 33 per cent. In the ultracentrifuge it was polydisperse. The peak had an $S_{20,w}$ of 182 and there was a heavier tail. The molecular weight of the principal component was about 25,000,000.

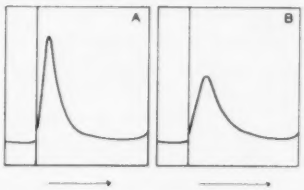

FIG. 5.—Ultracentrifugation of glycogen formed by phosphorylase plus "branching enzyme." Picture A, 20 min; B, 35 min, 13,410 rpm, 25°. $S_{20,w} = 49.8 \times 10^{-13}$.

Summary.—Highly purified phosphorylase is capable of forming an amylose

TABLE 1

AMYLOSE CHAINS FORMED IN *DE NOVO* REACTION

	Time of incubation (hr)	Average chain length	I_2-KI maximum* mμ	Intrinsic viscosity (ml gm^{-1})	Calculated molecular weight
Standards					
1	..	53	580	14.6	8,611
2	..	99	595	17.95	16,070
3	..	162	610	27.5	26,285
4	..	335	615	45.8	54,335
Products					
1	3	2049	655	186	331,900
2	6½	1285	650	130	208,200
3	30	1050	642	111	170,100
4	30	1377	642	138	223,100
5	29	2520	650	218	408,200

* The spectrum of about 25 μg/ml polysaccharide in the presence of 10 mg % I_2–20 mg % KI was read against 10 mg % I_2–20 mg % KI/ml of water in a cell with 1 cm light path. The observed extinction values at the maxima were from 26 to 36 per mg product (as glucose).

chain from glucose-1-phosphate in the absence of any primer. The chain does not have a phosphate group at its reducing end. The initial *de novo* act occurs very slowly and is characterized by an apparently absolute lag period during which no P_i is liberated and no exchange occurs with P_i^{32}. There follows a more rapid reaction of chain elongation leading to the formation of a product which may become more than 2,000 glucose units long. In the presence of highly purified amylo-1,4 → 1,6-transglycosidase the rate of polysaccharide formation is enormously increased, and the product of *de novo* synthesis is glycogen with an end group of 7.5 to 6.3 per cent and a molecular weight ranging from 2 to 25 million in different experiments. Phosphorylase and the "branching enzyme" together constitute a catalytic system for the *de novo* synthesis of glycogen, a process which could be of special importance during embryonal development.

* This work was supported in part by grant RG-4761 from the U.S. Public Health Service and in part by a grant from the Nutrition Foundation, Inc.

The following abbreviations are used: G-1-P, α-glucose-1-phosphate; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; 5′-AMP, 5′-adenylic acid; DEAE, diethylaminoethyl.

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[10] In repeated trials with different enzyme preparations the rate of the reaction was faster with the synthetic than with the commercial sample of G-1-P. The reason for this difference has not been discovered. It is possible that commercial samples of G-1-P contain one or more substances which are inhibitory and that synthetic G-1-P is free of these substances. α-Glucose-1,6-diphosphate was without effect on the system.

[11] The content of the latter enzyme was estimated by its effect on the iodine color of soluble starch in the presence of NaCl (10 min, 30°).

[12] We are grateful to Dr. T. J. Schoch for suggesting the use of this procedure.

[13] Kindly provided by Dr. Luis Glaser.

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*THE MECHANISM OF THE DE NOVO SYNTHESIS OF POLYSACCHARIDE BY PHOSPHORYLASE**

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In the preceding paper[1] it has been shown that phosphorylase is able to initiate the formation of an amylose chain from glucose-1-phosphate, the so-called *de novo* reaction. It has also been shown that in the presence of amylo-1,4 → 1,6-transglucosidase the *de novo* product is glycogen. In this paper are reported observations on the mechanism of the *de novo* reaction.

Formation of Short Chain Oligosaccharide Intermediates in de novo Synthesis.—The detection of short chain intermediates was made possible by the use of C^{14}-G-1-P (specific activity, 70,450 cpm per μmole, determined with a proportional, windowless, gas-flow counter).[2] The C^{14}-G-1-P contained no C^{14}-oligosaccharides detectable by elution of a paper chromatogram (Whatman No. 1) developed in butanol/pyridine/water (3:2:1.5). There was also no hydrolyzable glucose (3 hr, 1 *N* HCl, 100°) in excess of the amount of G-1-P measured microenzymatically *via* phosphoglucomutase and glucose-6-phosphate dehydrogenase. Thus, the C^{14}-G-1-P contained no polysaccharide which might influence the *de novo* reaction under study.

A reaction mixture containing C^{14}-G-1-P was prepared and incubated as described in Table 1, Experiment 1. Aliquots of the solution then were analyzed

TABLE 1

EFFECT OF α-AMYLASE AND OF A HIGH CONCENTRATION OF P_i ON *DE NOVO* SYNTHESIS

Identical reaction mixtures were prepared containing, initially, 1.43×10^{-2} *M* C^{14}-G-1-P, 5.75 mg per ml of phosphorylase *a* (0.046 μmole per ml of monomer), 1×10^{-2} *M* 5′-AMP and 0.014 *M* glycylglycine buffer, pH 6.8. In experiment 1 no further additions were made. In experiment 2, 220 μg per ml of crystalline salivary α-amylase was added. Experiment 3 differed from 1 by the addition of 11.2×10^{-2} *M* P_i. The values recorded are the changes produced in μm per ml, after incubation for 24 hours at 30° under toluene vapor.

Experiment No.	G-1-P	Glucose	C_2-C_6 oligosaccharides	Final ratio P_i/G-1-P
1	−1.07	+0.10	+0.052	3.37
2	−0.18	+0.19	+0.012	0.31
3	−0.11	+0.093	+0.001	8.61

microenzymatically for G-1-P and for free glucose. The reaction mixture gave a bluish color when tested with I_2; thus, maltosidic chains had been formed, presumably corresponding in amount to the disappearance of 0.97 μmole per ml of G-1-P. The final ratio of P_i to G-1-P (3.37) was that characteristic of the phosphorylase reaction at equilibrium at the prevailing pH.

Another aliquot of the final reaction mixture was spun in the analytical ultracentrifuge (rotor temperature, 30°). The protein peak was found to sediment at the rate characteristic of phosphorylase *a*. Centrifugation was continued until most of the protein was packed at the bottom of the cell. The rotor was decelerated slowly and a portion of the supernatant fluid carefully removed.[3] The pellet and some overlying supernatant fluid were recovered also. Analysis of the supernatant fluid showed that 80 per cent of the amount of protein originally present in it and 40 per cent of the radioactivity present as polysaccharide were removed by

ultracentrifugation. The centrifuged pellet was suspended in water and deproteinized by boiling. The resulting solution contained long amylose chains as shown by a bluish I_2 color. The ultracentrifuge supernatant fluid, the deproteinized ultracentrifuge pellet, and the deproteinized whole reaction mixture were subjected to chromatography on Whatman No. 1 paper for about 72 hr, using butanol/pyridine/water solvent (3:2:1.5). The position of each linear oligosaccharide from maltose to amylooctaose was revealed by a benzidine-trichloroacetic acid spray, using suitable reference standards. In no case was there enough of any of these oligosaccharides present in the experimental samples to be detected by the spray. The regions corresponding in position to the standards were eluted from each experimental strip by water and the amount of C^{14} in each eluate was measured. All interzones between oligosaccharide regions were also eluted to show that the radioactivity measured was confined to discrete areas on the chromatograms. Amylohexaose was the longest oligosaccharide whose radioactivity could be measured without interference from the C^{14}-G-1-P spot above it. The efficiency of water elution of each oligosaccharide with chain length from 2 to 6 was found to be about 90 per cent.

Table 2 shows the amount of each oligosaccharide present in the ultracentrifuge supernatant fluid (where 20 per cent of the phosphorylase *a* still remained), and

TABLE 2

FORMATION OF SHORT CHAIN INTERMEDIATES IN *DE NOVO* SYNTHESIS

Oligosaccharide	μMoles per ml Reaction Mixture*			
	In pellet with 0.037 μmole monomer	In supernatant with 0.0093 μmole monomer	Total with 0.046 μmole monomer	Per cent of total chains from n = 2 to n = 6
Maltose	0.0053	0.014	0.019	36
Amylotriaose	0.0084	0.010	0.019	36
Amylotetraose	0.0024	0.0061	0.0085	16
Amylopentaose	0.0018	0.0017	0.0035	6.7
Amylohexaose	0.0012	0.0016	0.0028	5.4
Total	0.019	0.033	0.052	100

* The composition of the reaction mixture is given in Table 1, Experiment 1.

the amount of each present in the ultracentrifuge pellet (with 80 per cent of the enzyme). The values for the latter have been corrected for the contamination of the pellet by the measured volume of supernatant fluid wetting it. It will be seen that every intermediate chain length between 2 and 6 is present in the reaction mixture, and that many more chains 2 and 3 glucose units long occur than those 4, 5, and 6 glucose units long. This is probably explained by the fact that in the *de novo* reaction chains shorter than amylotetraose are lengthened only very slowly, while those longer than the tetraose are rapidly elongated to amylose. In support of this explanation is the observation that the shortest chain which phosphorylase *a* can use as a primer in the conventional reaction with G-1-P is amylotetraose.

The data in Table 2 show that there was about 1 chain of polysaccharide (n = 2 to n = 6) present per monomer of phosphorylase *a*. About half the number of each of these chains was free, the other half was still bound to the phosphorylase protein, presumably at the site on the protein where the *de novo* reaction occurs, and sedimented with it in a centrifugal field. These data also permit the calculation that about 20 per cent of all the G-1-P reacting to form polysaccharide was found in chains from 2 to 6 glucose residues in length. Most of the remaining

product consisted of long amylose chains. Because G-1-P was limiting in this experiment the number of amylose chains formed was small. These are conditions which favor the accumulation of short chains. Furthermore, if elongation is slow, short chains may become detached from the enzyme before they have grown to an appreciable size. The formation of free glucose (Table 1) may have the same significance—that is, it may represent hydrolysis of the first product of the *de novo* reaction.

In one experiment phosphorylase *a* eluted from a DEAE cellulose column (0.047 μmole per ml of monomer) was incubated with 1.4×10^{-2} *M* amyloheptaose and 1×10^{-3} *M* 5′-AMP in 0.04 *M* glycylglycine buffer, pH 6.7, in the absence of P_i and G-1-P. In 24 hours at 30°, 1.5 moles of free glucose were formed per mole of monomer. This result suggests that a polysaccharide chain can act as a glucosyl donor in the *de novo* reaction.

Effect of Addition of Amylase to the de novo System.—The rate of P_i formation from G-1-P by phosphorylase *a* alone or in the presence of added "branching enzyme" is strongly affected by the addition of α-amylase to the system. An example is given in Figure 1. The addition of less than 0.0005 μg per ml of α-amylase had no perceptible effect on the rate of P_i formation. At concentrations between 0.001 and 0.01 μg per ml there was a slight acceleration of the running rate of the reaction, presumably due to the creation of new end groups by chain splitting. When 0.05 to 0.1 μg per ml of α-amylase was added to the system, there was no detectable P_i liberation during 4 hours of incubation.

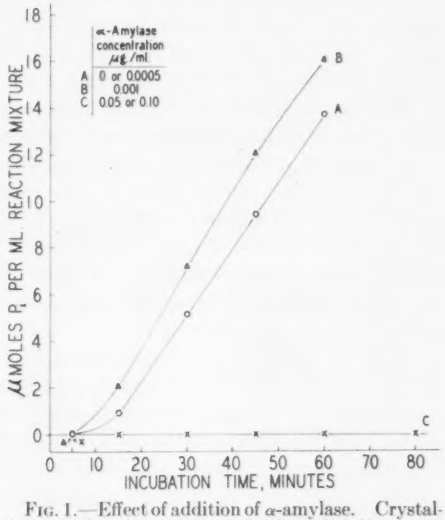

FIG. 1.—Effect of addition of α-amylase. Crystalline phosphorylase *a*, 92 μg/ml; "branching" enzyme, 2 μg/ml; 0.080 *M* G-1-P (Schwarz); no 5′-AMP; pH 6.7; 30°. Pancreatic α-amylase addition as indicated.

In other experiments the *de novo* synthesis of amylose by as much as 5 mg per ml of phosphorylase *a* in the presence of 5′-AMP was completely inhibited for as long as 24 hours by 200 μg per ml of salivary α-amylase. From the sensitivity of the P_i analysis method used, it can be calculated that no more than two moles of G-1-P could have reacted per mole of monomer of phosphorylase *a*. A similar result was obtained with crystalline sweet potato β-amylase. These two amylases have only one known action in common—the splitting of a maltosidic bond. The inhibition they produce when present in excess must be ascribed to this action whereby the *de novo* synthesis of polysaccharide is held in its slow phase.

That *de novo* synthesis does in fact proceed at a continuing slow rate in the presence of a large amount of salivary α-amylase was shown by the use of C^{14}-G-1-P. The composition of the reaction mixture used is shown in Table 1, Experiment 2.

After 24 hours of incubation the reaction mixture had no color when tested with I_2. By microenzymatic assay 1.25 μmoles per ml G-1-P remained in the solution and 0.19 μmole per ml free glucose had been formed.[4] The sum of the G-1-P remaining and of the glucose formed was essentially equal to the amount of G-1-P initially present. That oligosaccharide chains were formed (in amounts too small to be detected by measuring G-1-P disappearance) was shown by chromatography, elution, and counting of the radioactive oligosaccharide regions of the chromatogram (see above for details). The reaction mixture was found to contain about 0.01 μmole per ml of C^{14}-maltose and about 0.002 μmole per ml of C^{14}-amylotriaose. The amount of C^{14}-amylotetraose present (0.0002 μmole per ml) was barely detectable.

The results show that although chain growth beyond triaose is prevented, *de novo* synthesis *per se* cannot be suppressed by the action of large amounts of α-amylase. In the preceding paper an experiment was described in which incubation of G-1-P with phosphorylase *a* and a small amount of α-amylase (0.15 μg per ml) caused the disappearance of 98 per cent of added G-1-P and the formation of an achroic polysaccharide product with an average chain length of 2.7 glucose units. This result is qualitatively similar to that just described. The fact that nearly all of the G-1-P can react in *de novo* synthesis when α-amylase prevents chain growth implies a different equilibrium of the early part of the reaction, or a kinetic factor which makes the reverse reaction extremely slow, until chains at least 5 units long have been build up. A direct test of the ability of the *de novo* system to form oligosaccharide chains against a high P_i/G-1-P ratio was made in the experiment described below.

Effect of High Initial Concentration of P_i.—An incubation mixture containing C^{14}-G-1-P was prepared as described in Experiment 3, Table 1. After 24 hours of incubation the reaction mixture had no color when tested with I_2. Most of the G-1-P which disappeared was accounted for as free glucose. Chromatographic examination revealed, however, that there was a detectable amount of C^{14}-maltose (0.0003 μmole per ml), of C^{14}-amylotriaose (0.0005 μmole per ml), and of C^{14}-amylotetraose (0.0002 μmole per ml). Thus, even at a ratio of P_i/G-1-P = 8.6 net *de novo* synthesis was demonstrable. When compared with Experiment 1, the number of oligosaccharide chains formed per monomer of enzyme was very small; it is of interest, however, that the amount of free glucose formed was nearly the same in both experiments. If this represents hydrolysis of the first product of the *de novo* reaction, its rate of formation is apparently uninfluenced by a high initial P_i concentration. Because of experimental difficulties neither the reversibility of this reaction nor the position of its equilibrium has been tested. It seems clear from the data presented that chain elongation is largely prevented by a high initial P_i concentration, and that chains longer than amylotetraose cannot be demonstrated under these conditions.

Rate of de novo Chain Formation.—In Experiment 1, Table 1, there was formed about 1 polysaccharide chain per monomer of enzyme per 24 hours of incubation. Since the enzyme participates stoichiometrically in the reaction, a repetition of the *de novo* reaction requires that the chains become detached from the enzyme. In the ultracentrifugation experiment discussed above some chains were found to be detached and the formation of free glucose has been similarly interpreted. Further

evidence for a repetition of the reaction is given in Table 3. In these experiments the reaction was speeded up by the use of high concentrations of G-1-P, and it can be seen that after long incubation as many as 15 amylose molecules were formed per monomer of enzyme. When "branching enzyme" was added and the product formed was glycogen, the number of repetitive acts per active site of enzyme was even larger.

The first 2 experiments in Table 3 show that the *de novo* reaction is approximately linear with time. Again this suggests detachment of chains, otherwise the reaction rate would decrease exponentially with time. The calculated rate of chain initiation, equivalent to a reaction of 0.003 moles of G-1-P/mole active site/min, is extremely slow. Chain elongation, on the other hand, is nearly 2,000 times faster. These facts are interpreted to mean that each newly formed chain is immediately elongated to 1,000 to 2,000 glucose units, before it becomes detached from the enzyme. It should be noted that free amyloheptaose has a low affinity for muscle phosphorylase (K_m about 1.7×10^{-3} M). The much higher affinity of the *de novo* chains is attributed to the fact that they react in a bound form. This would also explain how the system can pass through maltose and amylotriaose, neither of which can act as primer for muscle phosphorylase.

TABLE 3

DE NOVO POLYSACCHARIDE SYNTHESIS*

Polysaccharide formed*	Time of incubation (hr)	Phosphorylase monomer (μmoles per ml)	Initial G-1-P conc. (μmoles per ml)	G-1-P reacted (μmoles per ml)	Molecular weight of product	No. of polysaccharide molecules per enzyme monomer
Amylose	3	0.039	86†	41.1	331,900	0.52
Amylose	6½	0.039	86†	63.4	208,200	1.3
Amylose	30	0.0057	200	75.6	170,100	12.6
Amylose	30	0.0057	200†	115	223,100	14.5
Glycogen	6½	0.00010	180	114	25,000,000	8
Glycogen	19	0.00023	150	118	2,240,000	41

* For additional details of preparation see Ref. 1.
† Incubation mixture contained 0.001 M 5′-AMP.

Participation of Apophosphorylase a in de novo Synthesis.—Apophosphorylase *a*, prepared as described previously,[5] was inactive in *de novo* synthesis when incubated with G-1-P at a level of 1.18 mg protein per ml for a period up to 6 hours (Fig. 2). When the resolved enzyme was added to an approximately equal amount of native enzyme, the lag phase was shortened and the final running rate was doubled. This effect is seen in the presence (curves B and C) and in the absence of 5′-AMP (curves E and F). Similar observations were made with phosphorylase *b* when it was added to phosphorylase *a* in the absence of 5′-AMP, conditions under which phosphorylase *b* alone is inactive. The meaning of these observations could be that the resolved enzyme (as well as phosphorylase *b*) still has the site where the *de novo* formation of amylose chains occurs. This would exclude pyridoxal-5′-phosphate itself as the point of attachment of the chains. The two covalent bonds between the native protein and pyridoxal-5′-phosphate also would seem excluded, since phosphorylase *a* reduced with $NaBH_4$ is active in *de novo* synthesis.

Discussion.—The problems posed by the *de novo* reaction are the following: (1) the nature of the initial act; (2) the mechanism of early chain growth, since maltose and amylotriaose, in contrast to amylotetraose, do not act as primers; (3) the repetitive nature of the initial act.

The lack of exchange between P_i^{32} and G-1-P in an early period of the *de novo* reaction, even with 100 mg of enzyme, speaks for an absolute lag or, at least, for an initial reaction which is kinetically irreversible.[5, 6] The meaning of an absolute lag period could be that since the *de novo* reaction is a special reaction, different from the ordinary phosphorylase reaction, it requires a structural change of the enzyme before it can occur.

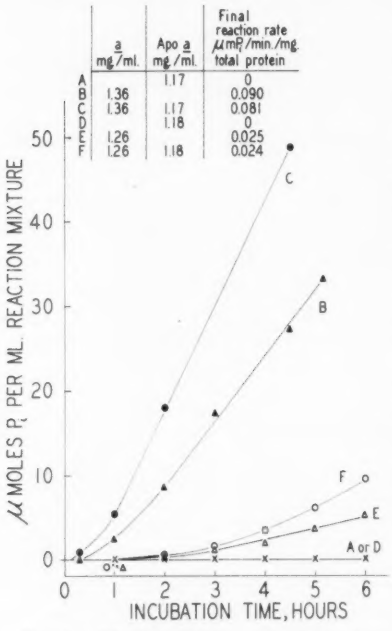

FIG. 2.—Effect of addition of apophosphorylase *a*. Curves A, B, and C, 0.085 *M* G-1-P; 0.001 *M* 5′-AMP. Curves D, E, and F, 0.039 *M* G-1-P; no 5′-AMP. Crystalline phosphorylase *a* and apophosphorylase *a* additions as indicated; pH 6.7; 30°.

Phosphorylase has been shown to split the C—O bond at carbon 1 of G-1-P.[7] This form of activation in the *de novo* reaction, where no other glucosyl acceptor is present, would lead to the formation of a glucosyl-enzyme which would then become the acceptor for chain growth. Experimental evidence for chain binding to the enzyme, at least for the short chains, has been presented in a preceding section. Owing to the lability of the glycosyl-enzyme complex, chains occasionally drop off, in which case the *de novo* reaction can become repetitive. This lability, which is indicated by the fact that the complex is split by boiling or by precipitation of the enzyme with alcohol, has so far prevented the identification of the glycosyl acceptor group of the enzyme.

The theory for the *de novo* reaction just presented leads to the question whether a glucosyl-enzyme complex is an obligatory intermediate in the usual phosphorylase reaction. If this were the case, the addition of C^{14}-glucose should lead to an exchange with both G-1-P and glycogen. This has not been observed.[6] The exchange with C^{14}-glucose has recently been retested, this time under the conditions of the *de novo* reaction, again with negative results. On the other hand, if in the *de novo* reaction a glucosyl-enzyme complex were formed once or twice per active center of enzyme and were followed by addition of glucose units with G-1-P as glucosyl donor, an exchange with C^{14}-glucose might be difficult to detect.

The *de novo* reaction is believed to occur in the following steps:

$$\text{G-1-P} + \text{E} \rightleftarrows \text{G-E} + P_i \tag{1}$$

$$\text{G-1-P} + \text{G-E} \rightleftarrows \text{G·G-E} + P_i \tag{2}$$

$$\vdots \qquad \vdots \qquad \vdots$$

$$\text{G-1-P} + (\text{G})_4\text{-E} \rightleftarrows (\text{G})_5\text{-E} + P_i \tag{3}$$

$$(\text{G})_n\text{-E} + \text{H}_2\text{O} \rightarrow (\text{G})_n + \text{E} \tag{4}$$

The initial stages of the *de novo* reaction have been observed to take place when the P_i/G-1-P ratio at the start of the reaction was 8.6, whereas the equilibrium ratio for the freely reversible part of the phosphorylase reaction at the pH of the system used is 3.4. This suggests that the initial stages are kinetically not easily reversible, and this is in agreement with the fact that amyloheptaose after prolonged incubation with large amounts of phosphorylase + P_i cannot be degraded further than to amylotetraose.[8]

Summary.—In the *de novo* synthesis of amylose by phosphorylase early intermediates of chain growth consisting of 2 to 6 glucose units attached to the enzyme have been demonstrated. Under optimal conditions the rate of synthesis was about one amylose chain per phosphorylase monomer (mol. wt. 125,000) per 6 hours. *De novo* synthesis has been shown to proceed at its slow initial rate even in the presence of α-amylase. In this case the products are small amounts of amylotriaose, maltose, and glucose. A smaller extent of *de novo* synthesis of maltose, amylotriaose, and amylotetraose can be shown in a system where the initial concentration of P_i is 0.01 M and the ratio of P_i to G-1-P is 8.6. Apophosphorylase *a* (with pyridoxal-5′-phosphate removed) is inactive in *de novo* synthesis. When this protein is mixed with the native enzyme, the inactivated enzyme apparently can be used as the site of attachment of the *de novo* chains. When native phosphorylase *a* is treated with $NaBH_4$ so that the bond linking pyridoxal-5′-phosphate to the enzyme becomes reduced, the enzyme still is active in *de novo* synthesis. These findings suggest that the attachment of the *de novo* amylose chain is to the enzyme protein and not to one of the functional groups of the coenzyme. Although in *de novo* synthesis enzyme-bound glucose presumably is an intermediate product, there is no evidence that a glucosyl-enzyme is an intermediate in the conventional phosphorylase reaction.

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The following abbreviations are used: G-1-P, α-glucose-1-phosphate; P_i, inorganic phosphate; DEAE, diethylaminoethyl; 5′-AMP, 5′-adenylic acid.

[1] Illingworth, B., D. H. Brown, and C. F. Cori, these PROCEEDINGS, **47**, 469 (1961).

[2] Kindly provided by Dr. Luis Glaser.

[3] The authors are grateful to Miss C. Lowry for her assistance in this experiment.

[4] The somewhat larger amount of glucose formed in this experiment can be attributed to the known action of α-amylase on amylotriaose which is split slowly to maltose and free glucose.

[5] Illingworth, B., H. S. Jansz, D. H. Brown, and C. F. Cori, these PROCEEDINGS, **44**, 1180–1191 (1958).

[6] Cohn, M., and G. T. Cori, *J. Biol. Chem.*, **175**, 89–93 (1948).

[7] Cohn, M., *J. Biol. Chem.*, **180**, 771–781 (1949).

[8] Brown, D. H., and C. F. Cori, in *The Enzymes*, ed. by P. D. Boyer, H. A. Lardy, and K. Myrback (New York: Academic Press, Inc., 1961), vol. 5, pp. 207–228.

*CRYSTALLINE LUCIFERIN FROM A LUMINESCENT FISH, PARAPRIACANTHUS BERYCIFORMES**

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Biochemical research on luminescent enzyme-substrate ("luciferin-luciferase") systems has resulted thus far in the isolation and crystallization of the luciferin from only two types of luminescent organisms, namely, the firefly[1] and the small ostracod crustacean, *Cypridina*.[2] Synthesis of the former luciferin has very recently been achieved,[3] and a tentative structure proposed for the latter luciferin,[4] which is a considerably more complicated molecule. Both differ chemically from the diffusible factors involved in bacterial luminescence.[5]

The present study has resulted in crystallization of luciferin from one of the two examples of luminescent fishes; namely, *Parapriacanthus beryciformes*[6] and *Apogon (marginatus) ellioti*,[7] which have yielded crude aqueous extracts of luciferin and luciferase that emit light on mixing at room temperature. The essential components of the two systems react interchangeably, and those of *Apogon* have been shown to cross-react with the luciferin and luciferase of *Cypridina*.[7] Present experiments have revealed that luminescent cross-reactions occur among all three of these systems.

A total of over 4,000 specimens of *Parapriacanthus* was collected at Tateyama,[8] Japan, and brought, packed in dry ice, to the Tokyo Kyoiku University, where the small, superficially embedded, ventral light organs were grossly removed and ground in a mortar chilled with dry ice. A very small amount of crystalline luciferin was obtained from such brei by the procedure of Shimomura *et al.*[2] as recently modified by Haneda *et al.* for crystallizing *Cypridina* luciferin,[9] involving initial extraction with methanol at low temperatures (circa −20°C) and chromatographing a butanol solution of the de-fatted residue on an alumina column. A much larger quantity, amounting to about 20 mg of pure crystalline luciferin, was obtained by the same procedure from the pyloric caeca dissected from some 2,300 specimens (Fig. 1).

The anatomical and histological details of the caeca remain to be worked out, but they appear to be connected by a minute duct to the light organs, and they evidently store luciferin. The intact caeca are nonluminous, and likewise the brei resulting from grinding with water, but a brilliant luminescence results on addition of *Cypridina* luciferase to the brei. In the stomachs of about a dozen of the 2,300 specimens, a few individual *Cypridina*, dead but still luminous, were discovered. This fact, plus observation of a luminescent reaction of crystallized luciferin from the fish with purified luciferase of *Cypridina*, and chemical similarities found between the luciferins from the two different sources (see below) raises the question as to whether *Parapriacanthus* depends on ingested *Cypridina* as the chief or sole source of its luciferin. While this possibility cannot be rigidly excluded on the basis of present evidence, it would present a fantastic situation

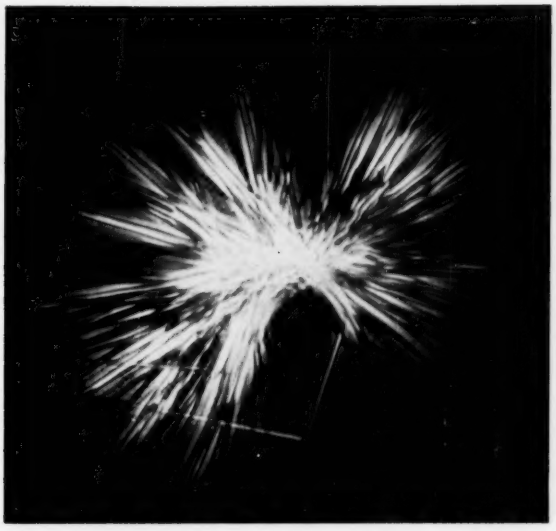

FIG. 1.—Luciferin isolated from the pyloric caeca of *Parapriacanthus*, recrystallized one time and photographed in immersion oil by means of a 50 ×, Wild varicolor phase lens. Magnification: × 1,200. Photomicrograph by L. I. Rebhun.

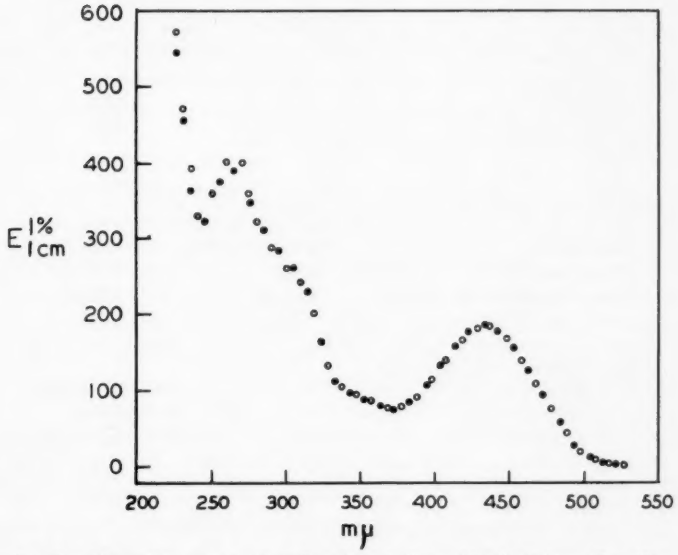

FIG. 2.—Absorption spectra of methanolic solutions of luciferin from *Cypridina* (hollow circles) and *Parapriacanthus* (solid circles) at 25°C.

from a biochemical and evolutionary point of view. Moreover, luminescent specimens of *Apogon*, whose light-emitting system anatomically does not differ greatly from that of *Parapriacanthus*, have been recently collected (by Y. H.) in areas of the Indian ocean where no specimens of *Cypridina* could be found.

The chemical properties of crystalline luciferin from *Parapriacanthus* and *Cypridina*, respectively, were found to be essentially identical in the following respects: (1) melting point, 185–195°C, not sharp, with evidence of softening and reddening at 170°C; (2) absorption spectra (Fig. 2), with $E^{1\%}_{1cm}$ (25°C, 435 mμ) measured as 187.0 and 187.5 for *Cypridina* and *Parapriacanthus*, respectively, suggesting fundamentally the same structure of the chromophore, as well as molar equivalence, probably also molecular weight; (3) specific luminescence potency in the reaction with *Cypridina* luciferase, amounting to 4,640,000 L.U. (light units, on an arbitrary scale of integrated total light) per mg for *Cypridina* luciferin, and 4,810,000 L.U. for *Parapriacanthus* luciferin; (4) a positive Sakaguchi reaction, indicating the same structure at a mono-substituted guanidine in the two luciferins; (5) identical paper chromatograms of the two luciferins, using Tsuji's solvent,[10] modified to consist of ethyl acetate, butanol, ethanol, and water in a ratio of 3:2:2:3; and (6) identical paper chromatograms of the products of acid hydrolysis (4 N.HCl, 15 hours at 120°C in air) and alkaline hydrolysis (10% barium hydroxide, 15 hours at 120°C in air), the former revealing the presence of isoleucine and 4-guanidino butyric acid, and the latter, the presence of isoleucine and 4-aminobutyric acid, using in each case a developing solvent consisting of butanol, acetic acid, and water in the proportion 4:1:1.

Evidence for chemical identity or near-identity of these luciferins is not surprising in view of their cross-reactions with the opposite luciferases, though this phenomenon is virtually unique among the luciferin-luciferase systems which have been separated from about a dozen different types among the numerous luminescent organisms known.[7, 11] While the significance from the viewpoints of comparative biochemistry and evolution remains to be elucidated, the foregoing results are interesting in constituting the third example of crystalline luciferin that had been isolated from a luminescent organism.

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*THE IDENTIFICATION OF A RIBOSOMAL-BOUND β-GLUCOSIDASE**

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Considerable evidence has accumulated in recent years to support the concept that ribosomes are the primary sites of protein synthesis. This view was originally supported by experiments *in vivo* and *in vitro* with C^{14} amino acids demonstrating that proteins pass through ribosomes during the course of their synthesis (see review by Novelli[1]). More recently, not only has a nascent precursor of soluble proteins been recognized on ribosomes,[2–4] but also specific protein[5–7] synthesis has been obtained with *in vitro* experiments in which the specificity for protein synthesis resides in the ribosomes.

Similar evidence has recently been reported for yeast. Protein synthesis is associated with particulate RNA.[8] Analysis of the ribosome fraction from exponentially growing yeast has revealed one major component having a sedimentation coefficient of 80S.[9, 10] In experiments with intact cells, C^{14} amino acids and S^{35} are incorporated first into ribosomal protein[11, 12] and can be chased from these to the soluble proteins.[12] Webster[13] has demonstrated *in vitro* that amino acids are incorporated into ribosomes in a manner analogous to that of mammalian and plant systems. Finally, the classes of S^{35}-labeled proteins released from ribosomes are identical to the classes of soluble proteins present in the cytoplasm.[14]

The essential feature to emerge from the above findings is that ribosomes are involved in determining the amino acid sequence for each particular enzyme. One would therefore expect that at any given moment a fraction of these particles would still be associated with newly synthesized enzyme. A measure of the number of these ribosomal-bound enzymes per cell might therefore provide a minimal estimate of the number of specific enzyme-forming sites (ribosomes) for a given enzyme. In the present communication, we have undertaken an examination of ribosomal particles from a yeast constitutive for β-glucosidase. The preparation of ribosomal-bound enzyme, a description of its properties, and an estimate of its cellular distribution are given.

Materials and Methods.—A diploid yeast (*Saccharomyces dobzhanskii* 1974 × *Saccharomyces fragilis* 110), constitutive for β-glucosidase synthesis, was grown aerobically at 30°C in synthetic

medium.[15] Growth was measured by following the optical density at 600 mμ in a Beckman Spectrophotometer or by direct count using a Levy hemocytometer. The yeast were harvested during exponential growth, washed twice with cold 6×10^{-4} *M* Mg cacodylate buffer, pH 7.2, and finally suspended in the same buffer (about 50 mg dry cells/ml). The cell suspension was passed through a French press[16] in the cold two to five times until examination under the phase contrast microscope showed that over 95 per cent of the cells were broken. The suspension was centrifuged at 4°C in the Servall centrifuge at 11,000 × *g* for 20 min. The precipitate was resuspended in buffer, recentrifuged, and the supernatant fractions combined. The precipitate remaining is referred to as the debris fraction. The supernatant fraction was centrifuged in the preparative Spinco at 105,000 × *g* for 2 hr, yielding a pellet of ribosomes. The ribosomes were suspended in cacodylic buffer and recentrifuged at 11,000 × *g* for 20 min to remove denatured material. Purified 80*S* ribosomes were isolated by centrifugation in an exponential sucrose density gradient employing a swinging bucket head rotor.[3]

β-Glucosidase was measured by following the hydrolysis of p-nitrophenyl-β-D-glucoside (PNPG) at 400 mμ[15] in either *M*/15 phosphate buffer, pH 6.8 (E^{M}_{1cm} 9.6 × 10³), or 0.005 *M* Mg-cacodylic buffer, pH 6.8. When the soluble enzyme was assayed in cacodylic and phosphate buffers, the ratio of activities was 1.40. Ribosomes, however, were stable in the cacodylic buffer but were broken down in the phosphate buffer. When *M*/15 phosphate buffer, pH 6.8, was used to measure ribosomal-bound enzyme, controls containing ribosome without substrate were included to correct for optical density measurements due to changes in light scattering from breakdown of the ribosomes. In a number of experiments in which enzyme was measured over 6-hr periods or longer, streptomycin (66 μg/ml) and penicillin (66 μg/ml) were included to prevent bacterial contamination.

Protein was measured by the method of Lowry *et al.*[17] using crystalline serum albumin as the standard. Ribonucleic acid (RNA) was determined by the orcinol method.[18]

Cacodylic acid was obtained from the Fisher Scientific Company. PNPG and carbohydrates were obtained as previously described.[19] Deionized glass-distilled water was used for all experiments.

Results.—(*a*) *Content of ribosomes per cell:* Chemical analyses were carried out on cells harvested in the logarithmic phase of growth in synthetic medium. At optical densities of 0.40 to 0.65, aliquots of 500–800 ml were removed for chemical analysis and 300 ml for dry-weight determinations. The cells were harvested by centrifugation, washed with cold buffer, and disrupted as described in *Materials and Methods*, and the various fractions were analyzed for protein and RNA. Table 1 summarizes the results of several experiments.

TABLE 1

DISTRIBUTION OF RNA IN YEAST*

	Direct count, cells/ml	Dry weight, mg/ml	Protein, mg/ml	RNA	
				mg/ml	per cent
Cell suspension	5.6×10^7	0.650	0.305	0.085	100
Debris†	0.28×10^7	...	...	0.0195	23.0
Soluble	...	...	...	0.0072	8.5
Ribosomes‡	...	...	...	0.0592	68.5

* Calculated on the basis of a cell suspension of OD = 1.000.
† The debris was removed by centrifugation at 11,000 × *g* for 20 min and washed twice with cold buffer.
‡ Ribosomes were collected by centrifugation at 105,000 × *g* for 1 hr.

The dry-weight content of protein (47%) and RNA (13%) of the diploid is characteristic of that found in numerous strains of yeast.[20] It can be seen that the bulk of the RNA (77%) is liberated upon rupture of the cell suspension. In this extract, 89 per cent of the RNA is associated with the ribosomal fraction and 11 per cent is soluble. Similar RNA distributions have been reported in yeast by Osawa.[21] About 22–24 per cent of the total RNA remains associated with the debris fraction, in various experiments. The number of intact cells in this fraction

ranged from 2 to 5 per cent of the original suspension; a value which is too low to account for the residual RNA. Since the debris fraction contains the cytoplasmic membranes, an attempt was made to determine if ribosomal particles were attached to these. Extended sonic oscillation or treatment of the debris fraction with lipase and proteinases liberates over 95 per cent of the debris RNA, approximately 30 per cent of which can be recovered as ribosomes. This same treatment leads to extensive degradation of ribosomes; thus, the actual content of ribosomal RNA in the debris is considerably higher. Therefore, between 78 and 91.5 per cent of the total RNA is ribosomal. Assuming that the debris RNA is representative of the total cellular RNA, the content of ribosomal RNA is about 1.5×10^{-12} gm/cell.

Ribosomes for chemical analysis were washed by repeated centrifugation (5 times) in the Spinco ultracentrifuge at 105,000 $\times$ g for 2 hr. A preparation, which showed a single 80S peak in the analytical centrifuge, had an E_{260}/E_{280} ratio of 1.89, a dry weight of 0.0908 mg per unit E_{260}, and an RNA content of 40 per cent of the dry weight. The chemical composition showed very little change on further recentrifugation. Chao and Schachman[9] and Yin[10] have observed similar results and have further calculated a molecular weight of yeast 80S ribosomes as 4.1×10^6 to 4.5×10^6. From the data reported here, the number of ribosome particles per cell of the diploid was calculated to be about 5.5×10^5.

(*b*) *Ribosomal-bound β-glucosidase:* Yeast cells containing constitutive β-glucosidase were broken in the French press and the 105,000 g pellet was layered on a sucrose density gradient[3] and spun at 45,000 $\times$ g for 180 min, and then the distribution of enzyme activity and 260 mμ absorbing material was measured. The results are shown in Figure 1. Most of the enzyme is associated with the soluble protein which is not sedimented under the conditions employed, whereas only a fraction of the activity is observed with ribosomes of the 80S class. The majority of the β-glucosidase activity of the initial ribosomal pellet (99.2%) is thus attributed to contamination by the soluble protein fraction.

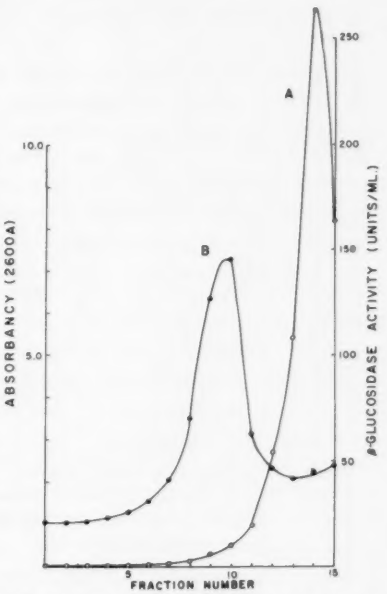

FIG. 1.—Sedimentation analysis of cell extract. The cell extract was overlayered on a continuous sucrose density gradient (5–20 per cent). Centrifugation 120 min at 45,000 $\times$ g. Curve A shows the β-glucosidase activity and curve B the absorbance at 260 mμ.

The ribosomes from Figure 1 are not completely freed of contaminating soluble protein. The material from fractions 8–10 were combined and the ribosomes collected by centrifugation at 105,000 $\times$ g for 180 min. The pellet was resuspended, clarified by low-speed centrifugation, and relayered on a sucrose density gradient.

The content of β-glucosidase following repeated density gradient centrifugation is shown in Figure 2. For comparison, the curve expected if each successive centrifugation had the same efficiency of resolving soluble enzyme from ribosomes is shown for the conditions employed in this experiment. After the second density gradient centrifugation, the enzyme associated with the ribosome fraction deviates from the expected exponential dilution. Following centrifugation, a minimum level of 0.00525 enzyme units/E_{260} was observed. This value, which has been observed on a number of experiments, is not lowered by further washings.

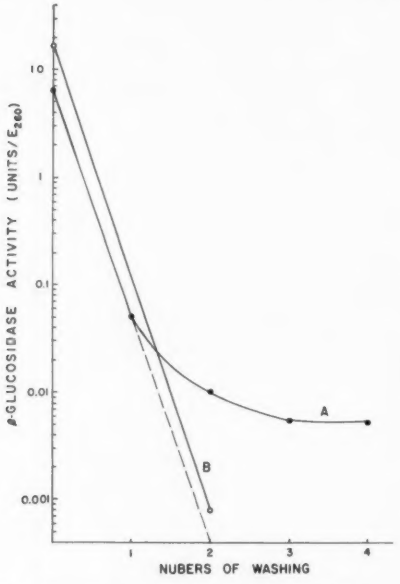

FIG. 2.—Changes of ribosomal β-glucosidase activity after repeated sucrose density gradient centrifugation. Centrifugation conditions are same as in Figure 1. Activities are expressed as enzyme units per E_{260}. Curve A represents the activity of ribosomes from β-glucosidase constitutive cells and curve B the activity of ribosome from β-glucosidase negative ribosomes mixed with supernatant fraction of constitutive cells to give the same activity level as constitutive extracts. The dotted line shows the theoretical dilution calculated from the conditions employed.

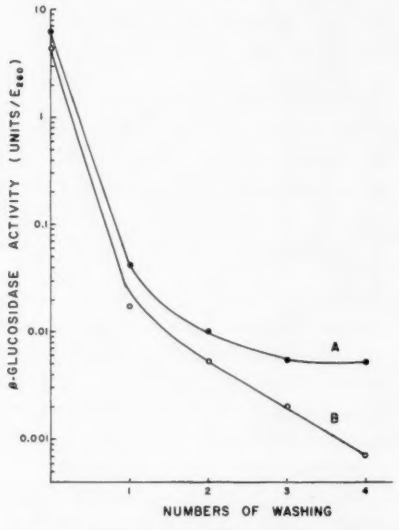

FIG. 3.—Effect of parafluorophenylalanine on the ribosomal-bound β-glucosidase. The cells were incubated with 0.05*M* p-FPA for 5 min. and washed, and the ribosome fraction was collected by centrifugation (B). Density gradient sedimentation conditions are the same as in Figure 1. For comparative purposes, the kinetics of washing of control ribosomes are also included (A).

As a critical test to eliminate the possibility of nonspecific adsorption as an explanation for the ribosomal-bound enzyme, the ribosomes of a β-glucosidase negative cell (*Saccharomyces cerevisiae*) were mixed with the soluble fraction of constitutive cells, separated by density-gradient centrifugation and analyzed. As can be seen in Figure 2, the loss of enzyme from the negative ribosome follows the theoretical exponential dilution. To eliminate the possibility that adsorption is a specific property of ribosomes from constitutive cells, a similar experiment was carried out in which S^{35}-labeled soluble proteins from β-glucosidase constitutive

TABLE 2

AFFINITY CONSTANTS FOR β-GLUCOSIDASE FROM VARIOUS SOURCES

Compound	Soluble* $\times 10^5$	Ribosomal $\times 10^5$	Released $\times 10^5$
PNPG (substrate)	8	8.5	9.3
Glucose	880	1,130	—
Phenyl-S-glucoside	330	330	430
4-Br-phenyl-S-glucoside	33	28	—
4-Bi-phenyl-S-glucoside	5.4	3.8	5.8
3-Cl-4-Me-Phenyl-S-glucoside	12	13	11

* Data from Hu *et al.*[15]

cells were mixed with unlabeled ribosomes from the same cells. When the mixture was subjected to the same washing procedure, all detectable traces of radioactivity were removed from the ribosomal fraction.

A direct test was undertaken to determine if the ribosomal-bound enzyme was a precursor of the soluble enzyme. Studies in which cells were briefly exposed to $S^{35}O_4$ or C^{14} amino acids indicate that a small fraction of the proteins of ribosomes has a transient existence of only a few seconds in the growing cell.[12] If the bound β-glucosidase were a portion of this nascent protein fraction, it should have a correspondingly brief existence on the ribosome. A method for measuring this was provided by the finding that the incorporation of p-fluorophenylalanine (p-FPA) into yeast proteins in the place of phenylalanine leads to linear growth and the synthesis of enzymatically inactive protein.[22] The incorporation of p-FPA into β-glucosidase leads to an inactive form of the enzyme,[23] and therefore one would expect that it would rapidly lead to the replacement of active ribosomal β-glucosidase by an inactive form of the enzyme. The following experiment was designed to test this possibility. P-FPA was added at a final concentration of 10^{-2} *M* to a growing culture of cells. After five min, three liter aliquots were removed, chilled with −20°C ice, washed, and broken in the French press, and the ribosomes were collected and subjected to successive density-gradient centrifugation as previously described. The β-glucosidase activity of ribosomes from the p-FPA-incubated cells behaves quite differently from that observed with the control material (Fig. 3). Although the initial activities were very similar, there was no indication of a constant specific activity in the p-FPA-incubated material. After four such centrifugations, the activity was less than 10 per cent of that observed in the control cells. When the final ribosomal fraction was analyzed in an analytical ultracentrifuge, only an 80*S* component was observed.

Although a dramatic decrease in the ribosomal-bound β-glucosidase was observed in the presence of p-FPA, there was no detectable change in the total level of soluble enzyme. Addition of 10^{-2} *M* phenylalanine rapidly gave rise to an active form of the bound enzyme; after 50 seconds, the ribosome fraction regained over 50 per cent of the normal level of active β-glucosidase. These experiments indicate that the β-glucosidase which is bound to ribosomes is a precursor of the soluble

TABLE 3

PHYSICAL-CHEMICAL PROPERTIES OF VARIOUS β-GLUCOSIDASES

Properties	Soluble	Ribosomal	Released
pH optimum	6.4–6.6	6.4–6.6	—
ΔH (cal/mol)	16,200	15,200	—
S_{20}	14	80	14.3

enzyme and further eliminate an *in vivo* nonspecific adsorption, since in the p-FPA experiments ribosomes essentially negative for β-glucosidase activity were isolated from cells having normal levels of soluble enzyme.

(*c*) *Characterization of ribosomal-released enzyme:* We have previously demonstrated that chelating agents release soluble protein from ribosomes and that the pattern of ribosomal-bound protein is identical to that found normally in the cytoplasm.[14] In attempting to determine if the soluble and ribosomal-bound β-glucosidase are identical, we have examined the specificity and physical-chemical properties of these as well as the enzyme released by 1 *M* borate. The specificity data, recorded as affinity constants for the substrate (PNPG) and other β-glucosides, are summarized in Table 2. Although these values range over 100 fold, from 4×10^{-3} *M* for phenyl-S-β-D-glucoside to 4×10^{-5} *M* for 4 bi-phenyl-β-D-glucoside, there is no significant difference between soluble, ribosomal, or released enzyme. A similar conclusion is evident from examination of the physical-chemical properties of these enzyme activities (Table 3). The pH optimum and ΔH are identical for the soluble and ribosomal enzyme. The sedimentation constants of the soluble and ribosomal enzyme were determined by moving boundary sedimentation; a partition cell was used to measure the sedimentation constant of the released enzyme. The ribosomal-bound enzyme sediments at the same velocity (80*S*) as the E_{260} material. The enzyme released from ribosomes has the same sedimentation value as the soluble enzyme. From these data, it seems reasonable that the ribosomal-bound enzyme is identical to the soluble enzyme.

(*d*) *Latent β-glucosidase:* The latency of a portion of the ribosomal-bound β-glucosidase was initially observed when enzyme activity was compared under conditions maintaining ribosome integrity (cacodylic buffer) and under conditions favoring ribosome degradation (phosphate buffer). The activities for the ribosomal-bound enzyme were 0.00525 units/E_{260} and 0.0133 units/E_{260} for cacodylic and phosphate buffers respectively. Since the ratio of the cacodylic to $PO_4^{=}$ activities at pH 6.8 for the soluble enzyme was 1.40, the corrected activity of the enzyme active in intact ribosomes is 0.00375 units (phosphate buffer)/E_{260}. Thus, only 28 per cent of the ribosomal-bound enzyme is active in intact particles. The remaining 72 per cent is latent and demonstrable only when the particles are degraded. Increases of 3- to 6-fold in β-galactosidase activity have been reported when ribosomes of *Escherichia coli* are treated with specific anti-β-galactosidase serum.[24] Noninduced, inducible cells contained about one molecule of ribosomal-bound β-galactosidase per cell.

(*e*) *Cellular distribution of β-glucosidase:* The number of total enzyme molecules per cell can be readily calculated from the differential rate of β-glucosidase synthesis and the properties of the purified enzyme. The molecular weight of the enzyme is 330,000 and its turnover number for PNPG in phosphate buffer, pH 6.8, is 13.2×10^4 enzyme units/min/mg protein.[15] Thus, from the Δ enzyme/Δ OD of about 240,[25] and from the data in Table 1, it follows that the total number of molecules of enzyme per cell is about 62,000.

Assuming (*i*) identical turnover numbers for all β-glucosidases, (*ii*) no more than 1 molecule of β-glucosidase per ribosome, and (*iii*) no selection of specific ribosomal types during isolation, the cellular distribution of enzyme can be estimated. From the above data and those of Table 1, there is one molecule of

ribosomal-bound enzyme per 6.9×10^5 ribosomes or 0.8 molecules per cell.

Discussion.—Numerous claims have been made for protein and enzyme association with the ribosome and microsome fraction. However, in a number of cases, either contamination from soluble protein or protein derived from other cellular components was found to be adsorbed on these particles either prior to or during their isolation. In the present example, such contamination must be seriously considered, since only a small fraction of the total β-glucosidase is bound to the 80*S* particles. For the following reasons, we believe that the binding observed in the present experiments represents the *in situ* situation: (*a*) 80*S* yeast ribosomes contain a residual β-glucosidase activity which cannot be removed by density-gradient sedimentation without disrupting the integrity of the particles; (*b*) in control experiments with S^{35} proteins or β-glucosidase-negative ribosomes, density-gradient sedimentation removes all activity from the ribosome fraction; (*c*) *in vivo* brief exposure to p-FPA gives rise to ribosomes essentially negative for β-glucosidase activity in cells containing normal levels of soluble enzyme. Similar studies have led to the identification of enzymes and specific proteins on ribosomes or microsomal particles. These include hemoglobin,[2] albumin,[26], amylase,[4, 27, 28] aldolase,[7] β-galactosidase,[24] RNase,[29–31] α-chymotrypsinogen,[4] peroxidase,[33] and antibodies.[34] Further, the proteins released by disruption of the particles from guinea pig pancreas,[4] bovine pancreas,[35] and yeast[14] closely resemble the soluble proteins from the supernatant fraction.

It seems reasonable to assume that the proteins released following disruption of ribosomal particles are primarily newly synthesized enzymes. Demonstrations from *in vivo* pulse-labeling experiments that the highest specific activity was associated with the proteins[3, 4, 35] and α-chymotrypsinogen[4] released from these particles support this view. We have previously reported that in yeast labeled amino acids are incorporated first into the 80*S* ribosome fraction and that upon addition of unlabeled amino acids, this label is transferred to the soluble protein fraction.[12] In the present experiments with β-glucosidase, the results with p-FPA, leading to the formation of inactive enzyme, lead to the conclusion that the ribosomal-bound enzyme represents newly synthesized material.

In the present experiments, approximately one molecule of ribosomal-bound β-glucosidase was found per cell. Since enzymic activity would appear only late in synthesis of nascent protein, one would expect the total number of templates specific for one enzyme to exceed the number of these templates retaining completed enzyme. An estimate of the total number of β-glucosidase templates per cell can be obtained from the following assumptions: (*i*) specific ribosomes are involved in the synthesis of the majority of the cellular proteins; (*ii*) all of the ribosomes are active in protein synthesis; (*iii*) for nonrepressed enzymes, the rate of peptide bond formation is constant for all proteins. Based on the molecular weight of 100,000 as average for yeast cell proteins, and on the data in Table 1, there are approximately 3.3×10^7 molecules of protein per cell. Thus β-glucosidase represents 0.18 per cent of the protein population. From this, it follows that about 100 ribosomes per cell participate in the synthesis of β-glucosidase. The number of β-glucosidase templates per cell therefore could range from a minimum of 1 to possibly 100.

The association of latent enzymes with ribosomal particles appears to be a

widespread phenomenon. RNase has been reported to be latent in particles from a variety of organisms,[29-32] including yeast.[36] Pancreas microsomes contain a latent amylase.[28] In *E. coli*[24] and in the results reported here for yeast, the majority of the β-galactosidase and β-glucosidase associated with ribosomal particles are latent. The activation of these enzymes following ribosomal degradation is presumably due to an increased availability of enzyme to substrate. The existence of these latent enzymes suggests that the newly synthesized enzymes have a finite period of existence on the ribosomal particle and are removed from their template by some rate-limiting processes. The presence of active and specifically associated forms of β-glucosidase on ribosomes further implies that there may be discrete stages in enzyme release from these particles. Whatever the mechanism of the release may be, the present results suggest that claims for enzyme synthesis with ribosomal particles should be treated with caution, since the conditions employed for amino acid incorporation frequently lead to ribosome degradation and activation of latent enzymes.

Summary.—The distribution of β-glucosidase has been analyzed in a constitutive strain of yeast (*Saccharomyces dobzhanskii* × *Saccharomyces fragilis*). There are approximately 62,000 molecules of enzyme per cell, of which one molecule of enzyme is firmly bound to the 80*S* ribosome particles. Approximately $^3/_4$ of the bound enzyme is latent and can be recognized upon disruption of the particle by metal binding agents. The enzyme released from the particles is identical to the soluble enzyme. Inhibition experiments with an amino acid analogue, p-fluorophenylalanine, suggest that the ribosomal-bound β-glucosidase is a precursor of the soluble enzyme.

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*AMINO ACID TRANSFER FROM AMINOACYL-RIBONUCLEIC ACIDS TO PROTEIN ON RIBOSOMES OF ESCHERICHIA COLI**

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We are continuing attempts to understand the mechanism by which peptide bonds are formed in protein synthesis. Progress depends largely on the characterization of the enzymic processes involved in polypeptide synthesis from the amino acid esters of soluble ribonucleic acid (sRNA), the biosynthetically active amino acids. In a previous study,[1, 2] we used the aminoacyl-sRNA microsome system of rat liver. The recently developed *E. coli* ribosome system[3, 4, 17] seemed to hold more promise. The present report deals with an analysis of the components of the *E. coli* aminoacyl-sRNA ribosome reaction.

Methods.—Preparation of ribosomes: *E. coli* B was grown in a Biogen (American Sterilizer Co., Erie, Pa.), using a medium composed of 1 per cent dextrose, 1 per cent yeast extract, 0.25 *M* potassium phosphate pH 6.5; it was continuously harvested in a refrigerated Sharples centrifuge at the end of logarithmic growth. The cells were washed once and stored at −20° in 100 gm batches of paste. Ribosomes were prepared as required from 100 gm of bacteria by grinding with 250 gm of alumina (Alcoa A-303) and extracting with 300 ml of 0.01 *M* Tris HCl, pH 7.4, and 0.01 *M* magnesium acetate. After centrifuging first at 10,000 × *g* for 20 minutes and then the super

natant at 20,000 × *g* for 40 minutes, the ribosomal fraction was spun down from the resulting supernatant fluid at 78,000 × *g* for 3 hours. The pellets were rinsed and lightly homogenized in 70 ml of 0.01 *M* potassium phosphate, pH 7.0, and 0.0005 *M* magnesium acetate. To deplete the ribosomes of transfer factor, this suspension was recentrifuged at 15,000 × *g* for 10 minutes and the particles were spun down from the supernatant fluid at 105,000 × *g* for 3 hours. This washing was repeated twice and the resulting ribosomes were then lightly homogenized in 0.01 *M* Tris HCl, pH 7.4, and 0.01 *M* Mg acetate, and stored at −20° in small batches. Such preparations retained activity for several weeks. Re-sedimentation of the ribosomes after suspension in 0.0005 *M* Mg acetate, 0.01 *M* phosphate results in a preparation with one-and-one-half times the activity of ribosomes maintained in 0.01 *M* Mg acetate, 0.01 *M* Tris HCl. We suspect that this is due to partial purification of the low Mg^{++}-resistant and highly active 70 S particles described by Tissières *et al.*;[4] preliminary studies by means of sucrose gradient centrifugation support this view.

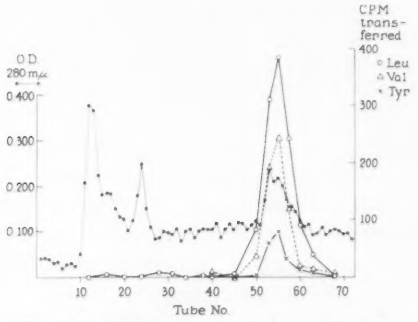


FIG. 1.—DEAE-cellulose column chromatogram of transfer factor. Application of 55 mg of the ammonium sulfate fraction was made to a 1.3 × 40 cm column equilibrated at 4° with 0.010 *M* potassium phosphate pH 7.4; 100 ml of 0.10 *M* potassium phosphate, pH 7.4, was passed through and a linear gradient of the same pH from 0.10 *M* to 0.25 *M* potassium phosphate started after tube 17. All buffers contained 0.004 *M* mercaptoethanol. Fraction volumes of 7 ml were collected and assayed for transfer factor with the labelled aminoacyl-sRNA's noted. Assay conditions were the same as given in Fig. 2, except as follows: with leucine and valine, 1.4 mg of ribosomal protein, 0.24 mg of sRNA (5000 cpm C^{14}-leucine, or 4040 cpm C^{14}-valine containing 1.23 × 10^7 cpm/μmole); with tyrosine, 3.5 mg of ribosomal protein, 0.57 mg of sRNA (1700 cpm C^{14}-tyrosine containing 5.2 × 10^6 cpm/μmole).

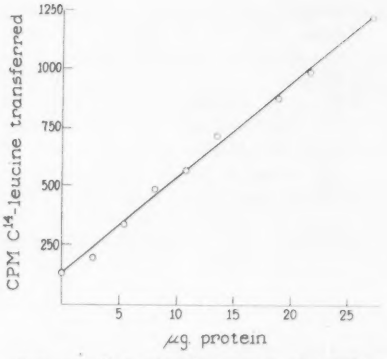


FIG. 2.—Concentration curve of transfer factor. The incubation mixture consisted of washed ribosomes (1.3 mg protein), 0.31 mg of sRNA charged with amino acids including 6660 cpm C^{14}-leucine (1.45 × 10^7 cpm/μmole), 0.0006 *M* GTP, 0.01 *M* PEP, 30 μg/ml PEP-kinase, 0.01 *M* GSH, 0.013 *M* $MgCl_2$, 0.03 *M* KCl, 0.05 *M* Tris HCl pH 7.4, and purified transfer factor as noted, in a volume of 0.50 ml. After 5 minutes at 30°, 5 per cent TCA was added, the precipitate extracted with 5 per cent TCA at 90° for 15 minutes, washed twice with TCA, once with 1:1 ethanol-ether, and counted in a windowless gas flow counter.

Preparation of sRNA charged with amino acids: *E. coli* sRNA was prepared by direct phenol treatment of the bacterial paste. After stripping of the sRNA by incubation in 0.5 *M* Tris HCl, pH 9, for 45 minutes at 36°, the sRNA was recharged with amino acids using the supernatant of alumina-ground *E. coli* as the enzyme source. For this purpose, the 105,000 × *g* supernatant fraction was treated with deoxyribonuclease and dialyzed against 0.02 *M* Tris HCl, pH 7.4, for 18 hours. A typical incubation mixture contained 3 mg of supernatant protein, 113 mg of sRNA, 0.0002 *M* of each of 21 amino acids including C^{14}-leucine, 0.003 *M* ATP, 0.01 *M* PEP, 30 μg/ml pyruvate kinase, 0.008 *M* GSH, 0.008 *M* $MgCl_2$, and 0.10 *M* Tris HCl, pH 7.2, in a volume of 4 ml. After incubation at 36° for 15 minutes, charged sRNA was re-isolated by phenol treatment and alcohol precipitation, and dialyzed against water. The concentration of sRNA was estimated by its optical density at 260 mμ, assuming 1.0 mg/ml equivalent to an optical density of 24.

Materials.—ATP, GTP, and CTP were products of Pabst Laboratories, Milwaukee. Phosphoenolpyruvate (PEP) silver barium salt and pyruvate kinase were obtained from C. F. Boehringer & Soehne, Mannheim, Germany. C^{14}-amino acids were from Volk Radiochemical Company, Chicago; and DEAE-cellulose was a product of Serva Entwicklungslabor, Heidelberg, Germany, and had a capacity of 0.74 mEq/gm.

Results.—Assay and Purification of the Amino Acid Transfer Factor.—Assay system: In preliminary experiments, it was found that in order to show the effect of supernatant fractions on amino acid transfer from sRNA to protein, the ribosomes had to be washed as described under Methods. Either 0.01 *M* phosphate, pH 7.0, and 0.0005 *M* Mg acetate, or 0.01 *M* Tris HCl, pH 7.4, and 0.01 *M* Mg acetate could be used for washing with equal effectiveness. With once-washed ribosomes, the supernatant fraction stimulated the transfer; with ribosomes

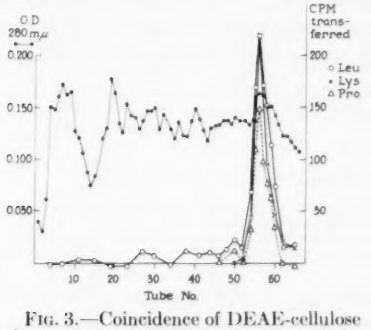


FIG. 3.—Coincidence of DEAE-cellulose eluate peak for different amino acids. From the *first ribosome wash*, 32 mg of the ammonium sulfate fraction was applied to a 1.3 × 40 cm column and protein eluted in 6 ml fractions as noted in Fig. 1, except that a linear gradient from 0.010 *M* to 0.25 *M* potassium phosphate pH 7.4 was used from the start. Transfer factor activity was assayed under conditions given in Fig. 2 except that sRNA was labelled with either C^{14}-leucine (0.28 mg sRNA, 3040 cpm), C^{14}-lysine (0.21 mg sRNA, 2350 cpm, 1.4×10^7 cpm/μmole), or C^{14}-proline (0.29 mg sRNA, 2050 cpm, 1.4×10^7 cpm/μmole).

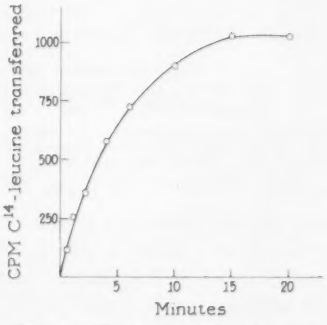


FIG. 4.—Time curve of leucine transfer from sRNA to protein. Incubation conditions as given in Fig. 2, except that the volume was 6.0 ml and contained ribosomes (20 mg protein), 7.2 mg of transfer factor, 5.0 mg of sRNA charged with amino acids including 51,000 cpm C^{14}-leucine. At each time point, 0.50 ml was pipetted into 5 per cent TCA and the precipitate treated as described in Fig. 2.

washed three times, an almost absolute requirement for supernatant appeared. This preparation then served for the assay of the transfer factor. The factor was found to lose activity completely after 1 minute at 70° and to be non-dialyzable. Its presence could be demonstrated not only in the 105,000 × *g* supernatant fraction, but also in the first ribosome wash. The wash fluid had twice the specific activity of whole supernatant, but only a small fraction of the total activity of the supernatant. The factor was partially purified from both sources by essentially similar procedures.

Purification.—After removal of nucleic acids with streptomycin, the solution was brought to 50 per cent saturation with solid ammonium sulfate, the pH being maintained at 7.4, and the precipitate was discarded. The precipitate obtained at 63 per cent saturation was dissolved in 0.01 *M* potassium phosphate pH 7.4,

with 0.004 M β-mercaptoethanol and was dialyzed against the same. This fraction was chromatographed on DEAE-cellulose as shown in Figure 1. Transfer factor activity was eluted at about 0.20 M phosphate concentration. On re-chromatography, the activity peak appeared in the same region of the chromatogram. Marked losses in activity occurred during chromatography and while the column fractions were kept at 4°, resulting in only 3 per cent over-all recovery of activity and 10–15-fold purification. When stored at −20°, however, the concentrated solution retained activity for several weeks. Leucine transfer with increasing amounts of the purified fraction is shown in Figure 2; maximum transfer occurred with 50 μg of protein.

Properties of the Purified Fraction.—Although the purified preparation showed some pyrophosphate exchange with ATP when the complete mixture of amino acids was added, the absence of leucine activating enzyme was shown by the failure of this fraction to transfer radioactive leucine to sRNA (Table 1). This excludes implication of the activating enzymes in amino acid transfer from sRNA. The

TABLE 1

TEST OF PURIFIED TRANSFER FACTOR FOR LEUCINE ACTIVATING ENZYME

Enzyme preparation	C^{14}-leu in sRNA (cpm)
105,000 × g supernatant, 0.30 mg	4,220
Transfer factor, 0.16 mg	58

Each incubation mixture contained: 2.0 mg of sRNA, 4×10^{-5} M C^{14}-leucine (3.7×10^6 cpm/μmole), 0.003 M ATP, 0.01 M PEP, 30 μg/ml pyruvate kinase, 0.008 M GSH, 0.008 M $MgCl_2$, and 0.10 M Tris HCl pH 7.2 in a volume of 0.25 ml. After 20 minutes at 36°, C^{12}-leucine and carrier RNA were added, and RNA precipitated by an equal volume of cold 1 N perchloric acid. The precipitate was washed four times with cold 0.5 N perchloric acid, once with 1:1 ethanol-ether, and counted.

question whether there is a general transfer factor was approached in the experiments shown in Figures 1 and 3. When the DEAE-cellulose column fractions were assayed with sRNA charged with different C^{14}-amino acids, a single identical peak of activity resulted whether C^{14}-leucine, lysine, proline, valine, or tyrosine was used. This is strong evidence in favor of a general transfer factor in contrast to the report of von der Decken and Hultin[5] whose data on differences between valine and tyrosine transfer in the rat liver system appear unconvincing.

Ribosome Specificity of Transfer Factor.—In a previous report[2] we noted that rat liver DOC-particles required a soluble factor partially purified from liver supernatant for transfer of amino acid from sRNA; there the supernatant fraction from rabbit, pigeon, chicken, or calf liver, or from rabbit reticulocytes could replace rat liver supernatant. *E. coli* supernatant, however, was found to be ineffective with rat liver DOC-particles and rat liver supernatant was without effect with *E. coli* ribosomes even though aminoacyl-sRNA of *E. coli* was used as amino acid donor in all cases (Table 2). Hence, the transfer factor has relative specificity for the particle preparation. Similar results have been obtained by Rendi and Ochoa.

TABLE 2

RIBOSOME SPECIFICITY OF TRANSFER FACTOR

Ribosomes	Supernatant fraction	C^{14}-leu trans. (cpm)
E. coli	None	90
E. coli	Liver	80
E. coli	*E. coli*	665
Liver	None	21
Liver	*E.* coli	19
Liver	Liver	244

Incubation conditions for *E. coli* ribosomes were the same as those in Figure 2, except as follows: 1.7 mg ribosomal protein, 0.26 mg of sRNA with 4,190 cpm C^{14}-leucine, 0.8 mg of *E. coli* supernatant, or 1.6 mg of rat liver supernatant. Conditions for rat liver DOC-particles as described previously.[2] *E. coli* sRNA was used with both ribosome preparations.

Properties of the Purified System for Transferring Amino Acids to Protein.—Figure 4 presents a time curve for amino acid transfer in the purified system. From a very early time, there is a fall in rate of transfer which is due largely to loss of amino acid from sRNA; addition of fresh amino acyl-sRNA after 10 minutes resulted in further amino acid transfer.

The cofactor requirement of the system is generally similar to that reported for mammalian preparations except for the high Mg^{++} concentration (0.012 *M*–0.016 *M* is optimal) (Table 3). Magnesium ion, however, can be replaced, at least partially, by spermidine which Cohen and Lichtenstein[6] have shown will replace Mg^{++} in stabilization of heavier ribosomes. In contrast to the liver microsome or DOC-particle system,[1] the effect of SH-compounds is not as striking, although GSH generally stimulates. Both puromycin[7] and chloramphenicol markedly inhibit transfer.

When sRNA charged only with C^{14}-leucine is substituted for fully charged sRNA, transfer is diminished by more than half, indicating that other aminoacyl-sRNA's are required for maximal transfer. Presumably this effect would be more striking

TABLE 3

COFACTOR REQUIREMENTS AND EFFECT OF INHIBITORS

Conditions	C^{14}-leu trans. (cpm)
Complete system	1,690, 1,760
− GSH[1]	1,420
− PEP, kinase, GTP	194
− GTP	319
− PEP, kinase	427
− added Mg^{++}[2]	87
− added Mg^{++}[2] + spermidine phosphate, 0.01 *M*	1,470
sRNA charged with C^{14}-leu, but no other amino acids	653
+ C^{12}-leu, 0.0008 *M*	1,630
+ Puromycin, 0.0004 *M*	46
+ Chloramphenicol, 0.00019 *M*	577
− Ribosomes	17

[1] Transfer factor dialyzed to remove mercaptoethanol.
[2] 0.0008 *M* Mg^{++} present from ribosome solution.
Conditions were the same as in Figure 2; 54 μg transfer factor was present in each tube.

TABLE 4

RETENTION OF ACTIVITY OF sRNA AFTER AMINO ACID TRANSFER

Pretreatment: Conditions	Pretreatment: Leu trans. to protein (%)	Charging of Re-isolated RNA: C^{14}-leu, cpm / 0.23 mg RNA	Charging of Re-isolated RNA: mμmoles leu / mg RNA
1. Complete system	27	4,650	1.05
2. Complete system + puromycin	1.9	4,900	1.09

Pretreatment: 4.4 mg of sRNA charged with amino acids, including 12,700 cpm C^{14}-leucine (3.74×10^6 cpm/μmole), ribosomes (40 mg protein), 4 mg of transfer factor, plus cofactors and salts as in Figure 2 were incubated in a volume of 4.0 ml for 10 minutes at 30°; in a second tube (subsequent No. 2), 0.0005 *M* puromycin was included. At the end of incubation, sRNA was recovered and stripped of amino acids as noted in the text. Recovered sRNA was tested for leucine acceptance as described in Table 1, except that 0.23 mg of sRNA was used and 2.7×10^{-5} *M* C^{14}-leucine was present with 1.93×10^5 cpm/μmole.

if purified leucine activating enzyme were used to charge sRNA instead of the crude dialyzed supernatant. Similar results with the liver microsome system were reported by Acs.[8] As shown in Table 3, addition of unlabeled free leucine does not affect the transfer of the sRNA bound leucine. In a separate experiment it was shown, furthermore, that free C^{14}-leucine, C^{14}-threonine, and C^{14}-proline were

not incorporated into protein with the purified system even when ATP was included.

Recovery of Active sRNA after Transfer.—Following incubation of aminoacyl-sRNA with ribosomes, the sRNA was recovered by phenol treatment of the incubation mixture and extraction of the alcohol-precipitated RNA with cold 1 *M* NaCl. This RNA was stripped of residual amino acids by incubation at pH 9 with 0.5 *M* Tris HCl at 36° for 45 minutes. It was then re-precipitated and dialyzed, and tested for acceptance of leucine and of AMP. A similar experiment was carried out with incubation in the presence of 0.0005 *M* puromycin which inhibits incorporation (Table 3). As shown in Table 4, sRNA which has functioned in amino acid transfer has the same activity for accepting leucine as the control. Moreover, recovered sRNA does not accept AMP end groups (Table 5), a result in agreement with findings in whole cells.[11, 12] These data indicate that sRNA remains intact and active after amino acid transfer, and functions as a cofactor in the over-all incorporation of free amino acids being successively charged with amino acid and discharged at the template.

Deacylation and Transfer.—In the experiment recorded in Table 4, the total loss of amino acid from the aminoacyl-sRNA after incubation with and without puromycin was determined, including a rather constant chemical hydrolysis. From these values together with the transfer of leucine into protein, the over-all balance of the amino acid that is liberated is computed. As indicated in Table 6, in addition to transfer there occurs a hydrolysis of amino acids from the RNA. What is particularly significant is that in the presence of puromycin this hydrolysis is larger by an amount similar to the transfer that was prevented by the puromycin. These observations prompted a further exploration of this apparently enzymatic hydrolysis. It appears from Table 7 to be dependent on the same factors that are operating in amino acid transfer into protein. The experiments were carried out in the presence of a puromycin concentration where transfer is inhibited and only hydrolysis is observed. This hydrolysis obviously has a relation to the transfer reaction, in particular since it increases, by blocking the transfer, comparably to inhibition. So far analysis of the hydrolyzed leucine has indicated it to be electrophoretically comparable to free leucine.

Comments.—Nature and generality of transfer factor: Since the transfer factor could be separated from the activating enzyme for the amino acid which it transfers, it appears that the activating enzyme is not part of the peptide linking system. Further proof for this may be seen in the non-specificity of the transfer factor. The activity for all amino acids tested, including leucine, valine, tyrosine, lysine, and proline, was found in the same rather sharp peak on elution from a DEAE-cellulose column. By implication we assume that the peptide linking enzyme does not carry specificity for amino acids.

The reasons for the apparent fragility of transfer protein fractions are not explained. It is not impossible, although no indications have been found so far, that we are dealing not with a single but rather with a multiple fraction. A recombination of various column fractions so far has not shown encouraging results. The function of GTP in the process and its possible relationship to the transfer factor are in urgent need of explanation, and we will return to this in a subsequent communication.

TABLE 5

TEST FOR INTACTNESS OF ADENOSINE TERMINAL OF RECOVERED sRNA

Source of sRNA	cpm / 0.23 mg RNA	mμmoles AMP / mg RNA
1. From complete system	83	0.90
2. From complete system + puromycin	83	0.90
3. Venom-degraded sRNA	2,500	27

The following were incubated in a volume of 0.30 ml at 36° for 30 minutes: 0.23 mg of sRNA pretreated as described in Table 4, 0.00016 *M* C^{14}-ATP (4.02×10^8 cpm/μmole), 0.01 *M* PEP, 30 μg/ml pyruvate kinase, 0.00016 *M* CTP, 0.008 *M* GSH, 0.10 *M* Tris HCl pH 7.5, 0.006 *M* $MgCl_2$, and 0.57 mg of a 0–40 per cent saturated ammonium sulfate fraction of *E. coli* 105,000 × *g* supernatant.[9] RNA was precipitated and washed as noted in Table 1 with C^{12}-ATP present. Venom-degraded sRNA was prepared by incubating sRNA with venom phosphodiesterase[10] at pH 8.8 and 35° until 4 per cent degradation occurred.

TABLE 6

BALANCE OF DEACYLATION OF AMINOACYL-sRNA WITH C^{14}-LEUCINE AS MARKER

	No inhibitor	0.0005 *M* puromycin
	Per cent original C^{14}-leucine	
1. Total loss	76	71
2. Transfer	27	2
3. Chemical deacylation	17	17
4. Enzymatic deacylation*	32	52

* No. 1—(No. 2 + 3).

TABLE 7

REQUIREMENTS FOR ENZYMATIC DEACYLATION OF AMINOACYL-sRNA

Conditions	Experiment 1		Experiment 2	
	cpm liberated	% enzymatic deacylation	cpm liberated	% enzymatic deacylation
Chemical hydrolysis	310	—	1,070	—
Complete system	780	26	2,680	39
− ribosomes	300	0	1,170	2.4
− transfer factor	400	5.0	1,400	7.9
− GTP, PEP, & kinase	300	0	1,620	13
Ribosome concentration doubled	1,050	41	—	—
Incubation time	10 min.	10 min.	15 min.	15 min.

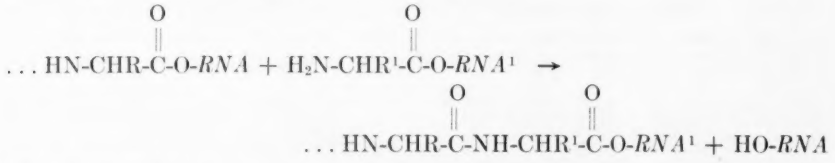
Incubation conditions as in Figure 2, except as follows: Experiment 1, 0.66 mg of ribosomal protein, 0.09 mg of sRNA, 0.0005 *M* puromycin in a total volume of 0.25 ml; Experiment 2, 4.2 mg of ribosomal protein, 0.18 mg of sRNA, 0.15 mg of transfer factor, 0.0005 *M* puromycin in a total volume of 0.50 ml. After incubation at 30°, RNA and protein were precipitated and washed as noted in Table 1, and the precipitate counted. In Experiment 1, 1,790 cpm were present in the precipitate at zero time; in Experiment 2, 4,180 cpm. Liberated cpm is the difference between the zero time and incubated values. Enzymatic hydrolysis is taken as total cpm liberated minus chemical hydrolysis.

Species specificity of transfer factor: In view of the interchangeability of aminoacyl-sRNA's derived from microbial or mammalian cells, it was somewhat surprising that the transfer factor, i.e. the peptide linking enzyme, displays a specificity for the ribosome on which the reaction takes place. The same aminoacyl-sRNA of *E. coli* may be used with different particles but to be joined into the peptide chain, *E. coli* ribosomes will respond only to *E. coli* factor, and mammalian ribosomes will respond only to mammalian transfer factor. The peptide linking factor, therefore, might relate to the protein part of the ribosome, which would most easily explain such a specificity.

Catalytic function of sRNA: The functional integrity of sRNA is maintained after discharging of amino acids on the ribosome; used RNA can be recharged and the accepting adenylic acid terminal remains untouched. In a protein synthesis cycle, therefore, RNA's act in a cyclic fashion as coenzymes which accept the matching amino acid on the activating enzyme, carry it to the microsome, and transfer it into a peptide link.

The meaning of the transfer of some sRNA to the ribosome[13, 14] remains to be

further explored. It would be expected that on every growing peptide chain the terminal amino acid carries its corresponding sRNA:[15]



This could explain the presence of a fraction of the sRNA's on the template.

Enzymatic deacylation of aminoacyl-sRNA and the effect of puromycin: The deacylation of charged sRNA in the puromycin-inhibited system seems to be one of the most promising observations made in the course of these studies. The dependence of this deacylation on the completeness of the system seems to indicate that one is dealing here with a degenerate reaction where, through the action of puromycin, hydrolysis partly takes the place of condensation. These results also provide some clues to the mechanism of inhibition by puromycin. We have found that puromycin acts directly on the ribosome, irreversibly and independently of transfer factor and GTP.[16] The deacylation experiments indicate, however, that the poisoned ribosomes which no longer transfer amino acids to protein are still active in enzymatic deacylation. This suggests that puromycin still leaves intact a partial reaction in aminoacyl transfer to protein, but rather specifically prevents the final condensation of the activated amino acids to peptides.

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† U.S. Public Health Service Postdoctoral Fellow.

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*RECOMBINATION DURING TRANSFORMATION IN HEMOPHILUS INFLUENZAE**

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In bacterial transformation, the irreversible uptake by a cell population of DNA extracted from a variant strain is followed by integration and expression of the variant "information" carried by the transforming DNA. Little is known of the details or mechanism of transformation after uptake of the DNA by a cell, i.e., of the process of integration which results in a permanent hereditary change. One must conclude from studies on linked transforming factors, however, that some process of recombination is involved.[1–3] The studies reported in this paper were designed to determine the time and some of the conditions for recombination during transformation.

Goodgal and Herriott have shown that a population of cells which has irreversibly taken up transforming DNA releases a large part of this activity when lysed shortly after uptake.[4] In the experiments to be described, the intracellular fate of transforming DNA has been followed quantitatively by this technique in the case of two linked markers located on the same DNA molecule.

Goodgal (3) has shown that in *Hemophilus influenzae* the two factors responsible for resistance or sensitivity to the antibiotics streptomycin and cathomycin (Novobiocin) are linked. When DNA is extracted from donor cells resistant to both cathomycin and streptomycin and is used to transform cells sensitive to both antibiotics, some 20 per cent of the resistant clones are endowed genetically with resistance to both antibiotics. The remainder of the transformations are to streptomycin resistance alone and to cathomycin resistance alone. The degree of linkage obtained depends to some extent on the method of preparation of the DNA and the conditions of transformation, but for material treated in the same way, the fraction of linked transformations is the same. The failure to retain complete linkage may be attributed to loss or inactivation of one or the other marker in the process of extracting the DNA from the donor cells, in the process of transformation itself, or in both processes.

One can transform to streptomycin or cathomycin sensitivity as well as to resistance. The frequency of transformation to sensitivity is the same as to resistance, and the same linkage relationships obtain.[3] Resistance and sensitivity thus may be considered as alleles of the same locus, the frequency of genetic incorporation of either or both factors being a function of the spatial relationships between the loci and not of the particular forms utilized in the test.[3] When, in the course of transformation, both streptomycin and cathomycin loci are genetically incorporated, linked transformations are obtained. When only one locus is genetically incorporated, transformations result which are recombinant with respect to the two markers in question, one marker supplied by the donor population and the other by the recipient population. This is the restricted manner in which the term recombination will be used in this paper, i.e., recombination between two linked loci on a single DNA molecule.

In the experiments to be described, cells marked by resistance to one antibiotic are transformed with DNA from cells resistant to the other antibiotic. At various times after uptake, samples of the recipient culture are lysed. Since lysis releases the total DNA content of the cells, both donor and recipient cell DNAs are released. Their transforming activities are then assayed on a double-sensitive strain. This technique enables one to follow the initial appearance and increase of linked transforming activities and affords insight into the sequences of intracellular events following DNA uptake. The results demonstrate that half the maximum amount of recombinant transforming activity, as measured by linkage of donor and recipient transforming factors, can be recovered from the recipient cell population within 15 minutes after a short uptake period. Furthermore, this genetic recombination occurs in the absence of either growth or appreciable increase in recipient transforming activity. Generally similar results were recently reported for pneumococcus by Fox and Hotchkiss.[9]

Materials and Methods.—Culture strains: Rd: A stock strain of *Hemophilus influenzae*, sensitive to less than 3 μg/ml streptomycin and to less than 1 μg/ml cathomycin. This strain was obtained originally from Alexander and Leidy.[8] S: A strain of Rd resistant to greater than 500 μg/ml streptomycin and sensitive to less than 1 μg/ml cathomycin, obtained by transforming into Rd a factor for one-step high-level streptomycin resistance. This marker was originally isolated by Alexander and Leidy.[8] C: A one-step mutant of Rd resistant to at least 2.5 μg/ml of cathomycin and sensitive to less than 3 μg/ml streptomycin.

Media: The growth medium used was a 50:50 mixture of Levinthal Stock and three per cent Eugonbroth (BBL), to which was added 2 μg/ml DPN.[5] For plating, a final concentration of 1.25 per cent Bacto-agar (Difco) was added. These media are referred to as Elev and Elev agar respectively.

For dilution of cells for plating, 3 per cent Eugonbroth (BBL) was used.

Transforming preparations: C DNA: A purified preparation of DNA[5] extracted from C (concentration of 0.5 mg DNA/ml). S DNA: Deoxycholate lysates of concentrated suspensions of S (approximate concentration of 0.02 mg/ml DNA).

Preparation of competent cultures: Highly competent cultures, i.e., cultures in which the majority of cells are capable of absorbing DNA, were grown by the aerobic-nonaerobic technique.[5]

Transformation frequencies of markers used: The frequency of transformation per unit of DNA to cathomycin resistance is twice that to streptomycin resistance.

Terms: Viable cells and transformed cells: the figures given for the number of viable cells or transformed cells refer to the number of colonies obtained upon plating, i.e., to viable centers of growth.

Experimental procedure: A competent culture of strain S containing approximately 1.5×10^9 viable cells per ml was diluted tenfold into Elev medium. An excess of C DNA was added and the mixture incubated at $36 \pm 1°C$ with gentle mixing for five minutes, after which time DNAase and Mg^{++} were added to give a final concentration of 1 μg/ml DNAase and 0.003 M Mg^{++}. Incubation of the culture was continued with shaking. After allowing two minutes for the DNAase to destroy the unabsorbed DNA and at intervals thereafter, samples were removed and used for the determination of the total number of viable cells, the number of transformed cells, and the preparation of cell lysates.

In the determination of the total number of viable cells per ml, samples were diluted appropriately, mixed with nonselective Elev agar, and incubated. The number of transformed cells per ml was detected by an agar layer technique: appropriate dilutions were mixed with nonselective Elev agar, and after two hours incubation to allow time for expression of the transformed characteristic, one set of plates was overlaid with agar containing cathomycin and another set with agar containing both cathomycin and streptomycin. After the overlayer had hardened, the plates were reincubated. The dilutions were selected to give about 200 colonies per plate.

Lysates were prepared from samples containing about 6×10^8 viable cells. The samples were immediately heated for 10 to 20 minutes at $63 \pm 2°C$ in order to stop the process of recombina-

tion. Such heating reduced the viability of the samples to 0.2 per cent of the original level or less. The heated samples subsequently were centrifuged, and the cells were washed with physiological saline containing 0.014 *M* sodium citrate and then resuspended in 2.5 or 3.0 ml of the citrate-saline fluid. Lysis was achieved by the addition of 0.16 ml of 2.0 per cent sodium deoxycholate and 0.2 ml of 1 *N* ammonium hydroxide. Following 15 minutes incubation, 0.2 ml of 1 *M* KH_2PO_4 was added to reduce the pH to approximately 8.[5]

Reciprocal experiments, in which competent C cells were treated with S DNA, were also performed following the same procedure outlined above.

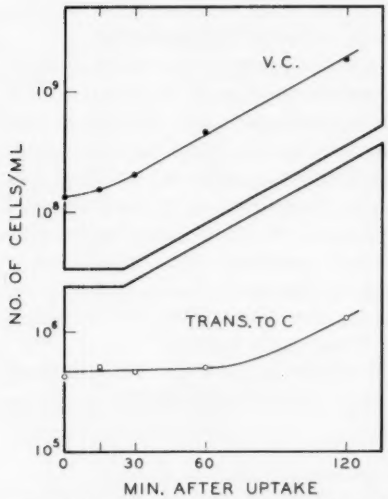

FIG. 1*a*.—Transformation of Recipient S Cells by C DNA. Closed circles are total number of viable centers (V.C.) and open circles are number of cathomycin-resistant viable centers (transformants).

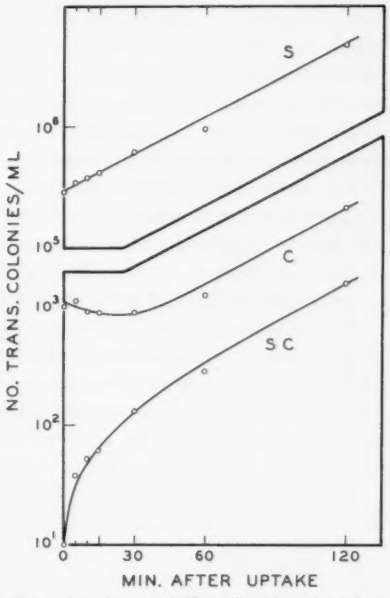

FIG. 1*b*.—Assay of Transforming Activity in Recipient Cell Lysates. *S*, streptomycin-resistant; *C*, cathomycin-resistant; *SC*, both cathomycin- and streptomycin-resistant. Assay mixture: 0.5 ml Rd + 4.45 ml 0.125 *M* saline + 0.05 ml lysate. To prepare the recipient cell lysates, 2.5 ml samples of the recipient culture were lysed at 0, 5, 10, 15, and 30 min. At 60 min, a $^1/_2$ dilution and at 120 min, a $^1/_{10}$ dilution of the recipient culture were lysed. In the graph, the 60-min and 120-min values have been multiplied by two and ten respectively. The *S* and *C* classes include the *SC* class.

The assay for transforming activity in the recipient cell lysates was performed by a slight modification of the routine transformation assay[6]—namely, by increasing the concentration of sensitive receptor cells in order to remove a larger part of the DNA from solution and thereby reduce the background of random doubles.[3] To 0.5 ml of competent Red cells diluted tenfold in 0.125 *M* saline, 0.05 or 0.1 ml of lysate was added, and the mixture was incubated for thirty minutes, after which time the numbers of transformations to streptomycin resistance, cathomycin resistance, and both streptomycin and cathomycin resistance were determined by the agar layer technique. The recovery of recipient transforming activity, i.e., the percentage of single cell equivalents in the lysates which gave recipient transformations in the assay, was 15 to 40 per cent, the

higher values being obtained when the recipient culture was C. The ratio of recovery of donor transforming activity to recovery of recipient transforming activity was high, closely approximating that expected on the basis of the number of cells transformed.

Experimental Results.—Recovery of recombinant transforming factor under conditions of normal growth of the recipient cell population: When a population of competent S cells was exposed to C DNA in Elev medium, the transforming activities present in the recipient cell population could be followed through several generations of growth. The results of such an experiment are presented in Table 1, in which the data from the assay are presented in the form of ratios, and in Figure 1, in which the numbers of transformed colonies obtained in the assay (1*B*) and the number of viable recipient cells and the number of transformed recipient cells (1*A*), are plotted as a function of time. Zero time in all experiments reported is two minutes after the addition of DNAase to the recipient cell population.

The data show that the recipient cell population, except for a possible slight lag, increased exponentially with the usual generation time of 30 minutes. The increase in recipient S DNA as measured by transforming activity, was proportional to the increase in recipient cells (Fig. 1). The recovery of donor C DNA remained constant or dropped slightly during the first 30 minutes after uptake (Fig. 1*B*), a fact indicating that there was either a lag in the replication of the introduced variant genetic information or that there was some loss of this information compensated for by a replication of the activity which remained. This lag showed up as a twofold drop in the donor/recipient ratio (C/S column, Table 1) during the first 30 minutes. By 30 minutes after uptake, the donor DNA was increasing at the same rate as the recipient DNA (Fig. 1*B* and Table 1, C/S).

A striking result was the early recovery of linked donor-recipient transforming activity (SC) from the recipient population (Fig. 1*B* and Table 1). Such linkage

TABLE 1

LINKAGE OF INTRODUCED C MARKER TO RECIPIENT S MARKER (ELEV):*
RATIO OF TRANSFORMANTS IN THE ASSAY OF RECIPIENT CELL LYSATES

Time (min)	SC/C	C/S	SC/S
0	0.82×10^{-2}	3.4×10^{-3}	0.28×10^{-4}
5	3.5×10^{-2}	3.1×10^{-3}	1.1×10^{-4}
10	5.9×10^{-2}	2.3×10^{-3}	1.4×10^{-4}
15	7.2×10^{-2}	2.0×10^{-3}	1.5×10^{-4}
30	15×10^{-2}	1.4×10^{-3}	2.1×10^{-4}
60	23×10^{-2}	1.2×10^{-3}	2.9×10^{-4}
120	23×10^{-2}	1.4×10^{-3}	3.2×10^{-4}

Note: The S and C classes include the SC class.
* Elev is the growth medium used (see *Materials and Methods*).

could be detected within a few minutes after uptake. Study of the recombinant to recipient transformation ratio (SC/S column, Table 1) shows that about half the maximum amount of recombination, as measured by transforming activity, occurred within 15 minutes after uptake. Linkage of donor and recipient transforming factors appeared to be nearly complete at 30 minutes after uptake and to be complete by 60 minutes after uptake, since by this time the recombinant to recipient transformation ratio had reached a nearly constant value.

Similar results were obtained when the reciprocal experiment was performed, i.e., when the recipient cells were C and the donor DNA was S (Table 2).

TABLE 2

LINKAGE OF INTRODUCED S MARKER TO RECIPIENT C MARKER (ELEV)*

Time (min)	Recipient Population: Viable centers	Recipient Population: S transformants	Ratio of Transformants, Assay: SC/S	S/C	SC/C
0	1.8×10^8	4.4×10^5	0.62×10^{-2}	2.6×10^{-3}	0.16×10^{-4}
5	...	...	2.8×10^{-2}	1.7×10^{-3}	0.48×10^{-4}
10	...	...	5.3×10^{-2}	1.4×10^{-3}	0.73×10^{-4}
15	...	...	8.3×10^{-2}	0.71×10^{-3}	0.61×10^{-4}
30	3.4×10^8	9.1×10^5	15×10^{-2}	0.69×10^{-3}	1.1×10^{-4}
60	6.5×10^8	1.1×10^6	21×10^{-2}	0.53×10^{-3}	1.1×10^{-4}
120	2.6×10^9	3.4×10^6	20×10^{-2}	0.66×10^{-3}	1.3×10^{-4}

Note: The S and C classes include the SC class.
* Elev is the growth medium used (see *Materials and Methods*).

Recombination in the absence of growth of the recipient population: To see whether linkage of donor and recipient transforming factor was dependent on growth of the recipient population, experiments were performed in saline where growth was sharply reduced. Growth of the recipient population was inhibited by centrifuging and resuspending a competent recipient culture in an equal volume of saline. Donor DNA was added to the resuspended cells, uptake was terminated after five minutes, and the culture was further incubated for 60 minutes. In the experiment for which data are presented in Figure 2 and Table 3, there was essentially no growth of the recipient cell population after uptake of DNA, as measured both by colony count and turbidity, and there was little if any increase in recipient cell transforming activity (top curve, Fig. 2). Nevertheless by 30 minutes after uptake, a considerable amount of linkage of donor and recipient transforming factors

TABLE 3

LINKAGE OF INTRODUCED S TO RECIPIENT C (SALINE)

Four ml of a competent culture of C cells were centrifuged and resuspended in 3.5 ml of 0.125 *M* saline and placed in a 36°C water bath. Then 0.3 ml of S DNA was added and five minutes later 2.5 μg/ml DNAase and 0.003 *M* Mg^{++} were added and the culture was incubated further, without shaking. At the times listed 0.3 ml samples were removed, diluted into 1.2 ml of citrate saline, and immediately heated for twenty minutes at 64°C. The heated samples were centrifuged, resuspended in 3.0 ml of citrate saline, and lysed as described in the text. The recipient culture was also sampled at the stated times for a determination of viable and transformed cells. Turbidity measurements were made on a Coleman Junior Spectrophotometer.

The recipient cell lysates were assayed for transforming activity as described in the experimental procedure. Two assays were performed using 0.1 and 0.05 ml of lysate respectively. The results of the assay are plotted in Figure 2. In the table and in the graph, the S and C classes include the SC class.

RECIPIENT CELL CULTURE

Time after uptake (min)	Viable centers	S transformants	SC transformants	Turbidity
0	1.4×10^9	8.4×10^6	3.6×10^6	0.18
30	1.5×10^9	1.4×10^7	4.0×10^6	0.19
60	1.5×10^9	1.1×10^7	3.2×10^6	0.185
120	1.2×10^9	7.6×10^6	1.9×10^6	...

ASSAY: RATIO OF TRANSFORMANTS

Time after uptake (min)	SC/S × 10^{-2}: 0.10 ml lysate	SC/S × 10^{-2}: 0.05 ml lysate	S/C × 10^{-3}: 0.10 ml lysate	S/C × 10^{-3}: 0.05 ml lysate	SC/C × 10^{-4}: 0.10 ml lysate	SC/C × 10^{-4}: 0.05 ml lysate
0	0.59	0.48	2.8	3.3	0.17	0.16
30	9.2	8.6	1.6	1.8	1.5	1.5
60	11	9.4	1.9	2.2	2.0	2.0
120	13	14	1.6	1.7	2.1	2.3

had occurred (bottom curve, Fig. 2, and SC/S and SC/C columns, Table 3). The results of this experiment have been confirmed by a reciprocal experiment in which the recipient S population after resuspension in saline was preincubated for 15 minutes before being exposed to C DNA. Once again there was no detectable

increase in viable count, turbidity, or recipient transforming activity of the recipient cells after uptake. By 60 minutes after uptake, the recombinant/donor DNA assay ratio was 0.16, a value which compares favorably with the ratio obtained for a similar time under conditions of growth (see Table 1).

Nature of the recombinant linkage: Evidence that recombinants do not represent a spurious linkage was obtained by the following procedure. The DNA from the recipient population was isolated and purified following uptake of donor DNA. The ratio of recombinant to donor transforming activity in the purified DNA was not appreciably altered from that obtained with crude material. In addition, this ratio was independent of the concentration of DNA.

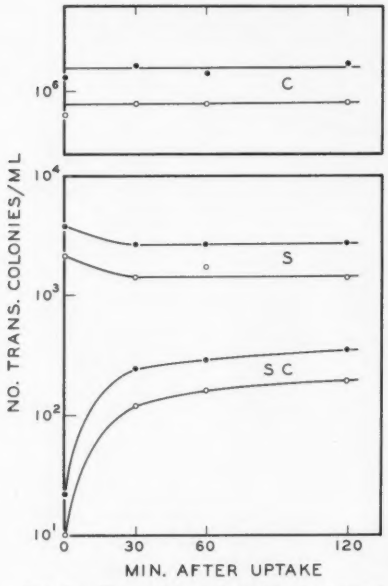

FIG. 2.—Figure 2 is explained in the Legend for Table 3. Open circles, 0.5 ml of lysate; closed circles, 0.10 ml of lysate. *S*, streptomycin-resistant; *C*, cathomycin-resistant; *SC*, treptomycin- and cathomycin-resistant.

A Sevag purification[7] of the recipient culture was performed following a 5-minute uptake period and 30 minutes' further incubation. Because of the small quantity of starting material, the recovery yield was low, constituting about 10 per cent of the total DNA in the cells. The ratio of U.V. absorption at 260 mμ to that at 230 mμ was 2.4, indicating that the DNA was not appreciably contaminated with protein, which absorbs highly at 230 mμ. The E260 and E230 values for purified DNA preparations given by Chargaff[10] are calculated to give ratios around 2.4, which value is obtained for purified DNAs in this laboratory. As shown in Table 4, a recombinant to donor ratio of 0.12 was obtained for the purified DNA. This value is close to those obtained for crude material under similar experimental conditions (Table 1). Furthermore, this ratio remained constant over a hundred-fold dilution range of the purified DNA preparation.

TABLE 4

CONSTANCY OF LINKAGE OF S AND C AT VARYING DILUTIONS OF NEWLY FORMED RECOMBINANT DNA

100 ml of a competent S culture was exposed to 0.1 ml of C DNA for five minutes at 36°C. DNAase was added and the culture was continued in incubation. 32 minutes after the addition of DNAase, the culture was heat-inactivated, and DNA was extracted from the cells by the Sevag procedure. 0.1 ml of the given dilutions of the extracted DNA was assayed for transforming activity by the method outlined under *Experimental procedure*.

DNA dilution	No. transformed colonies			Recombinant donor ratio
	S	C	SC	SC/C
2×10^{-1}	3.1×10^6	2.5×10^4	2.9×10^3	0.12
2×10^{-2}	7.6×10^5	7.1×10^3	8.2×10^2	0.12
2×10^{-3}	8.3×10^4	8.5×10^2	9.8×10^1	0.12

Note: The S and C classes include the SC class.

Discussion.—The process of recombination between linked loci in *Hemophilus*

influenzae takes place within a relatively short time after uptake of DNA by the recipient cells. The half-maximum amount of recombination is attained within 15 minutes after a short exposure of recipient cells to DNA. That the recombinant transforming activity found in the recipient cell lysates after uptake results from linkage of donor and recipient transforming factors inside the recipient cell and is not a result of events following lysis is shown by the following observations:

1. The recombinant transforming activity increases as a function of time after uptake even under conditions where there is little or no increase in donor or recipient transforming activities.

2. The recombinant activity, present 30 minutes after uptake in the crude DNA extracts (lysates), is retained during purification.

3. Upon dilution of crude or purified recipient cell lysates, the ratio of recombinant to donor, or to recipient, transformations remains constant.

If the recombinant transforming activity found in the recipient cell lysates resulted from agglomeration of DNA molecules in solution, such activity should have been lost in purification and in dilution of the lysate. If the recombinant transformations obtained in the assay were the result of random double transformations, their number should vary as the product of the frequency of the single transformations to streptomycin and cathomycin resistance, i.e., as a square function.[3] As noted above, both kinetic and dilution considerations eliminate this possibility. A small number of random double transformations is expected under the conditions of the assay. However, they represent a significant portion of the SC transformations only when the number of recombinants is still low (0 minute lysates).

Under conditions of growth it was found that by 30 minutes after uptake, donor transforming factor was increasing at the same rate as recipient transforming factor. This was surprising, since in the *Hemophilus influenzae* transformation system there is a characteristic lag of two to three generations before the number of transformants in the recipient population begins to increase (Fig. 1*A*). Only the initial delay in increase of transformants following uptake must be due to lag in the formation or increase of donor DNA replicas. Some of the additional lag after this time may be attributed to the fact that the viable center of growing cultures consists largely of two joined cells.[5] This would mask an initial increase in the number of transformed cells, since the daughter progeny would tend to remain together and give rise to only one resistant clone. If in addition it is assumed that there are two nuclei or half units per cell, only one of which is altered by the incoming DNA, the entire delay in increase of transformed recipient viable centers would be adequately accounted for.

We conclude from the experiments performed in saline that recombination can occur in the absence of growth and with less than 15 per cent synthesis of genetically functional DNA. This DNA was determined by measuring the transforming activity of the recipient cells. No more than 15 per cent of such synthesis should have escaped our attention by this procedure. Since increase in total DNA and increase in transforming activity are closely correlated,[6] it is clear that a large fraction of the recombination which occurs under conditions of growth also occurs when not more than 15 per cent of normal DNA synthesis is occurring.

Whatever mechanism of recombination might be proposed, it is clear that a

normal level of DNA synthesis is not necessary for that process. Two possibilities suggest themselves: (1) that the mechanism of recombination is a conservative one, with little or no concomitant DNA synthesis, or (2) there occurs a special kind of synthesis, restricted primarily to DNA molecules which have penetrated the cells and a portion of the DNA inside the cells.

The results indicate that transforming factors provide a most fruitful approach to the study of genetic recombination on the molecular level.

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*PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION OF TYPES 4 AND 5 ADENOVIRUS-SOLUBLE ANTIGENS**

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It has become increasingly apparent in many systems that the interaction of animal virus and host cell results in the production of antigenic materials readily separable from virus.[1–5] Although in most instances little is known about the nature or origin of these antigens, evidence has been presented in at least two instances which may be interpreted to indicate that certain of these materials are related to infectious virus, either as virus precursor materials,[6] or alternatively, as materials released by the degradation of newly synthesized virus.[7]

A primary obstacle to a more thorough study of such antigens as regards their relationship to the virus synthetic process has been the problem of separating these materials, first from normal cell components, and secondly, one from the other, so that each could be characterized independently of the others.

Adenovirus-infected HeLa cells offer a particularly good model for the study of virus-induced antigens for two reasons: (1) infected cells synthesize several easily identifiable products of infection (soluble complement-fixing antigens and a toxin-like material) in addition to infectious virus,[8-11] and (2) separation of these antigens by a simple procedure can be accomplished.[12-15]

The objectives of the study to be described were threefold: (1) to devise a technique of separating and isolating virus-induced antigens of type 5–infected HeLa cells in order to obtain them in a highly purified state, (2) to identify and characterize these antigens by immunological means, and (3) to compare the antigens associated with type 5–induced infections with those produced in type 4–infected cells. Type 4 virus was chosen for purposes of comparison because in terms of cytopathology, kinetics of synthesis, and neutralization by specific antiserum, this agent is markedly different from type 5 virus.[16] During the course of this study, Klemperer and Pereira[13] employed similar methods to effect the partial separation of antigens from types 2 and 5 adenovirus–infected HeLa cells.

Materials and Methods.—Tissue culture: Epithelial cells of the HeLa (Gey) strain and, on one occasion, of the Hep #2 strain[17] were employed throughout. These cells were propagated either in 32-ounce prescription bottles with Eagle's basal medium containing 10 per cent human serum or in screw-capped 14 × 150 mm test tubes with 40 per cent human serum in Hanks' balanced salt solution (BSS) by methods described elsewhere.[18]

Viruses and virus infections: Prototype strains of adenovirus types 4 and 5 were employed. Virus pools were prepared by infecting bottles as previously described.[18,19] Infected cells were incubated at 36°C until cytopathic effects were complete (5 to 6 days), at which time the cells were recovered from maintenance mixture by centrifugation at 1,000 rpm for 20 min and resuspended in phosphate buffered saline (PBS) at one-tenth the original volume. Resuspended cells were frozen and thawed 6 times and cell debris removed by centrifugation at 10,000 rpm (6,600 × *g*) for 15 min in a Spinco Model L preparative ultracentrifuge. The resultant supernatant material, hereafter referred to as crude virus, was stored at −28°C until used.

Virus infectivity and toxin titrations: Infectivity titrations were carried out in tube cultures of HeLa cells using $10^{-0.5}$ (1:3.2) dilution increments as previously described.[18] Toxic activity was assayed in normal HeLa cells prepared in the same manner as those used for infectivity titrations.[18] Serial 2-fold dilutions of the material to be tested were made in Hanks' balanced salt solution. Two-tenths ml of each dilution was added to duplicate tubes of HeLa cells which were incubated at 37°C. Six hours later, these cultures were examined in comparison with normal control tubes for evidence of cytopathic alterations. Changes which ranged from clumping and separation of the entire cell sheet from the glass surface to a rounding up of the cells at the periphery of the cell sheet only were arbitrarily scored from 5^+ to 1^+; the latter degree of cytopathology was designated as the titration endpoint. The reaction increased very little in severity from the 6th to the 20th hour, at which time changes attributable to virus multiplication often became evident.

Complement-fixation titrations: Various eluates obtained from DEAE columns were tested as complement-fixing antigens by means of a standard test employing approximately 1.5 exact units of complement.[20] Heat-inactivated (56°C for 30 min) type-specific rabbit antisera or convalescent human sera were employed according to the aims of the experiment. An antibody excess was used in all antigen titrations. Veronal buffer containing 0.005 *M* $MgCl_2$ and 0.0015 *M* $CaCl_2$ was employed as diluent throughout.[21] Titers were expressed as the highest initial dilution of antigen giving complete fixation.

Neutralization titrations: Neutralization titrations were carried out as described elsewhere.[22] The end-point was expressed as the highest final dilution of serum in the serum-virus mixture which prevented cytopathic changes in one-half or more of the HeLa cells in a culture tube. With two exceptions, toxin neutralization tests were carried out in the same manner as were the virus neutralization tests:[22] first, rather than virus, an amount of toxin sufficient to cause extensive cytopathic alterations was added to each tube of HeLa cells; and secondly, the serum-toxin mix-

ture was incubated at 36°C for 6 hr rather than for 6 days as in the virus neutralization test. Titers were expressed as the highest final serum dilution which completely suppressed cytopathic changes in the cultured cells.

Preparation of antisera: Rabbits were given 3 intramuscular injections of antigen in Freund's adjuvant at weekly intervals followed a week later by one intraperitoneal injection of antigen without adjuvant. Animals were bled 12 days after the last injection. Sera were separated by centrifugation, inactivated at 56°C for 30 min, and stored at −28°C.

Agar diffusion tests: The agar gel techniques employed were modifications of the double-diffusion plate technique of Ouchterlony.[23] Large plates, 9 cm in diameter, were used for the double-diffusion tests. Twenty-five ml of Ionagar[24] at a concentration of 0.6 per cent in water was used for each plate. All wells were cut 6 mm in diameter and the spacing between cups was 8 mm. Prior to use, a single drop of agar was placed in each well to prevent leakage. Specific precipitates were visible within 24 to 48 hr following incubation at room temperature.

Chromatographic procedures: Thoroughly washed DEAE cellulose[25] was used to make columns 1.1 cm in diameter and from 3 to 7 cm in height, depending upon the amount of protein to be added. DEAE columns were packed under pressure and equilibrated with 0.01 *M* phosphate or acetate buffer of the desired pH prior to the addition of the sample. Following this, a program of step-wise elution employing solutions of constant pH and increasing molarity was carried out. Such solutions were prepared by adding solid NaCl to 0.04 *M* buffer of the desired pH. Elution was carried out under 5 psi of air pressure at 2 to 4 ml of effluent per min. Following the collection of a suitable number of samples at each molarity, the column was washed with buffer of the same molarity in an amount equivalent to 10 times the volume of the original sample. In this manner, loss of resolution due to "smearing" of the eluting fractions was minimized. Eluates so obtained were tested for infectivity, toxicity, and the ability to fix complement.

Determinations for DNA, RNA, and protein: The Burton[26] modification of the diphenylamine reaction for deoxyribose was employed for the determination of DNA. Thymus DNA was used as a standard. RNA was determined by the orcinol method,[27] which measures ribose. D-Ribose was utilized as the standard. The method of Lowry *et al.*[28] was employed to determine protein concentration with crystalline bovine albumin employed as a standard.

Results.—Separation of the virus-induced antigens of type 5 virus on DEAE cellulose at pH 7.2: Homogenates of type 5 adenovirus-infected cells were adsorbed to DEAE as described under *Methods* and eluted with increasing concentrations of NaCl in 0.04 *M* phosphate buffer at pH 7.2. Infectivity, complement-fixation, and toxin titrations were undertaken with each eluate. Both human convalescent and type-specific rabbit antisera were employed in the complement-fixation tests. Figure 1 illustrates the characteristic and highly reproducible elution patterns exhibited by the antigens from type 5 adenovirus–infected cells. It is notable that in contrast to homotypic rabbit antiserum (Fig. 1*a*), human convalescent serum failed to reveal the presence of the complement-fixing (CF) antigen which eluted maximally with 0.08 *M* NaCl (Fig. 1*b*). This antigen will be termed the "early-eluting or E antigen," whereas the material which eluted maximally at 0.26 *M* NaCl will be called the "late-eluting or L antigen." The data graphically summarized in Figure 1*a* indicate that the toxin and the E antigen, collectively termed the E-T complex, may be separate and distinct entities. This will be considered in more detail in a later section. The elution characteristics of type 5 virus, toxin, and CF antigens as revealed by rabbit immune serum are similar to those recently observed by Klemperer and Pereira with types 2 and 5 adenoviruses.[13] These investigators, however, presented no data on the reaction of the soluble CF antigens with human convalescent serum.

When the eluates comprising the L antigen and virus peaks were pooled separately, redialyzed and chromatographed one or more times under the original

conditions, the elution characteristics of these moieties remained unchanged. This procedure led to a considerable improvement in the homogeneity of the antigens comprising each fraction. In this manner, it was possible to obtain antigenic materials suitable for preparation of immune sera. It should be emphasized, however, that under the conditions described repeated chromatography of the eluates comprising the E-T complex failed to effect a further separation of these antigens.

Immunological characterization of the antigens of type 5–infected HeLa cells: Specific antisera to the L antigen and the E antigen-toxin (E-T) complex were prepared in rabbits with Freund's adjuvant. Prior to use, each antigen preparation was rechromatographed three times and centrifuged four times at 105,000 × *g* for 120 min to remove contaminating antigens and residual virus. No infectious virus was detected in the final preparations of purified antigen. Antiserum to the E antigen-toxin complex will be referred to as "anti-E-T," whereas that to the L antigen will be termed "anti-L." Anti-5, anti-4, etc. refer to antiserum produced in response to homogenates of infected cells of the virus type designated by the number and may be assumed to contain antibodies to the "soluble" antigens in addition to neutralizing antibody for the virus type designated. Antiserum to homogenates of normal HeLa cells was also prepared and is designated anti-normal cell extract (anti-NCE).

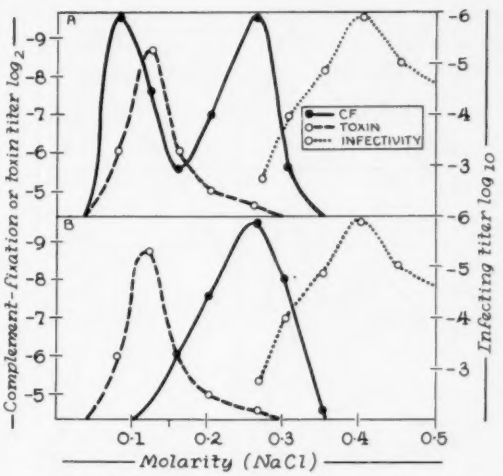

FIG. 1.—Fractionation of crude homogenates of type 5–infected HeLa cells on DEAE cellulose by gradient (stepwise) elution. Figure 1*a* illustrates the elution pattern obtained when homotypic rabbit antiserum was employed in the complement-fixation test; Figure 1*b* when convalescent human serum was used to test the same eluates for complement-fixing antigens.

Eluates obtained from chromatography of type 5 virus were examined for their ability to fix complement with various antisera. Figure 2*a* illustrates the characteristic type 5 elution pattern that was obtained when type-specific rabbit antiserum was employed for the complement-fixation tests. For purposes of orientation, the elution curve of toxin is included. The L antigen of type 5 adenovirus reacted strongly with antibodies specific for heterologous virus types; the use of antisera specific for types 2 and 4 virus are shown as examples (Figs. 2*b* and 2*c*). These results imply that the L antigen is the adenovirus group antigen, an interpretation which is further supported by the observation that antibodies in human serum reacted only with the L antigen (Fig. 2*d*). In contrast, type-specific rabbit antiserum reacted strongly with the E-T complex as well as with the L antigen (Fig. 2*a*). These data suggest that the E-T complex contains a type-specific antigen, as has been previously suggested by Klemperer and Pereira.[13]

These results provide a basis for the observation that type-specific rabbit antisera, unlike human convalescent serum, may be employed to type-specific adenoviruses by means of the complement-fixation technique.[8, 29, 30] Rabbit antisera react strongly with the type-specific antigens of the various adenoviruses, whereas human serum reacts only with the group-specific adenovirus antigens.

Since sera to heterologous virus types reacted, to a limited extent at least, with the eluates comprising the E-T complex (Figs. 2*a* and 2*c*), it may be assumed that this material contained a cross-reacting component in addition to the type-specific antigen previously mentioned. It seemed possible that the toxic moiety may represent this cross-reacting antigen; indeed, neutralization titrations employing various type-specific antisera and toxin preparations derived from different adenovirus types indicated that toxin behaved in a manner expected of a common antigen (unpublished data).

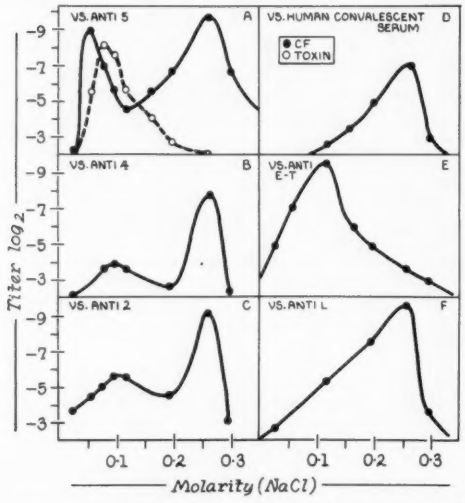

FIG. 2.—Complement-fixation patterns revealed by the reaction of various antisera with eluates obtained from the stepwise chromatography on DEAE of a crude homogenate of type 5 adenovirus–infected HeLa cells.

The reaction of antigen-specific antisera with the antigens of type 5–infected HeLa cells is shown in Figures 2*e* and 2*f*. There is relatively little cross-reactivity between these serum preparations, indicating that the chromatographic separation of E-T complex and L antigen was relatively complete.

It was possible to identify certain of the adenovirus-induced antigens by their precipitation reactions in agar with antisera from immunized animals. Eluates containing the E-T complex and the L antigen were tested in comparison with crude types 4 and 5 viruses against antiserum to unfractionated type 5 virus (anti-5). The results of this experiment are shown diagrammatically in Figure 3. Crude type 5 virus characteristically exhibited three well-defined lines of precipitate when reacted with homotypic rabbit antiserum. In contrast, crude type 4 virus preparations showed only a single sharp line when tested with the same antiserum. The type 4 precipitin band was continuous with that of the L antigen as well as one of the type 5 lines and may be considered to represent the group-specific or common adenovirus antigen. This observation is in agreement with the complement-fixation data regarding the immunologic characteristics of the L antigen. The weak diffuse zone of precipitation immediately adjacent to the center-well can be seen to represent normal cell antigen and was often encountered when crude antigen preparations were employed—in this case types 4 and 5 virus antigens. The two lines exhibited by the E-T complex were continuous with the first and second of the type 5 virus

lines but, unlike the L antigen, had no counterpart in the reaction of type 4 virus with type 5 antiserum. It would appear from this that one or both of these antigens, which presumably represent the toxin and the E antigens are type-specific. In the case of the E antigen this observation is compatible with complement-fixation data which would appear to indicate that this antigen is type-specific (Fig. 2). The toxin, however, seems to be a group rather than a type-specific antigen on the basis of cross-neutralization tests (unpublished data). It is pertinent to note at this point that infection with type 4 virus results in the production of only small quantities of toxin. The apparent type-specificity of this antigen as revealed by agar diffusion may therefore be a quantitative phenomenon.

When antisera prepared against the E-T complex and the L antigen of type 5-infected HeLa cells were tested against crude types 4 and 5 viruses, the results (Fig. 4) were compatible with the hypothesis that the L antigen was a group antigen common to type 4 as well as type 5 virus and that the E-T complex was apparently type-specific (also illustrated in Fig. 3). The result is, of course, subject to the same interpretation as discussed in the preceding paragraph.

To determine which of the two precipitin lines of the E-T complex was associated with the toxin, crude type 5 virus preparations were reacted with an amount of trypsin sufficient to destroy all traces of toxin activity. When trypsin-treated type 5 virus was tested against type 5 antiserum in parallel with untreated virus, the first of the E-T lines (Fig. 4) was not present. The results of this experiment are illustrated in Figure 5. This evidence made it possible to identify with reasonable surety the various precipitin bands associated with crude type 5 virus preparations (Fig. 5).

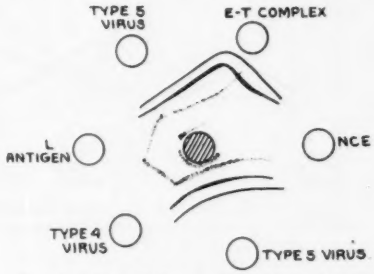

FIG. 3.—Use of the agar double-diffusion technique to reveal the relationships of various antigens. The center well contains antiserum to crude, unfractionated type 5 virus. NCE designates normal HeLa cell homogenate.

Chromatography of type 5 virus–infected cell lysates at pH 5.2: The preceding experiments suggested that the E-T complex consisted of two separate antigens. Therefore, a more satisfactory separation of this fraction was required to obtain a clear picture of the immunologic nature of the antigens. Assuming that the differences in the absorptive capacity of these antigens might be increased at pH values closer to their isoelectric points,[31] experiments designed to explore this possibility were undertaken.

Infected cell lysates were dialysed against 0.01 *M* acetate buffer at a pH of 5.2. The heavy precipitate which formed during this procedure did not contain detectable complement-fixing antigens and was discarded. The supernatant fluid was placed on DEAE columns previously equilibrated with an identical buffer. Eluates obtained with increasing NaCl concentrations in 0.04 *M* acetate buffer at pH 5.2 were titrated for toxin, infectious virus, and complement-fixing antigens. Various antisera were used in the latter tests. Figure 6 illustrates the results of a representative experiment. Under acidic conditions, the bulk of the E antigen passed through the column unadsorbed and was recovered in the original effluent and, to a

lesser extent, the 0.01 *M* acetate wash. The L or common CF antigen also exhibited a shift to the left in its elution characteristics and was recovered maximally in the 0.16 *M* NaCl eluate. In contrast, toxin was more strongly adsorbed to the exchange resin under acidic conditions than it had been at pH 7.2. Because of this, toxin was eluted with the L antigen rather than with the E antigen as had been the case at pH 7.2 (Fig. 1*a*). The identities of the CF antigens were verified by testing each of the eluates both with convalescent human serum (Fig. 6*b*), which has been shown to react only with the L or common antigen, and with specific rabbit antiserum to the L antigen (Fig. 6*c*) and to the E-T complex (Fig. 6*d*). The reaction of the L antigen-toxin complex, which eluted maximally at 0.16 *M* NaCl with anti E-T (Fig. 6*d*) suggests that the reaction of toxin with specific antiserum also resulted in fixation of complement.

Chromatography of E antigen-toxin (E-T) complex at pH 5.2: Although chromatography of crude type 5 virus at pH 5.2 presented a method of obtaining E antigen

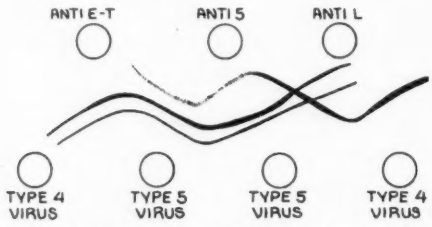

FIG. 4.—The reaction of certain antigen-specific antisera with crude types 4 and 5 virus as revealed by agar-diffusion.

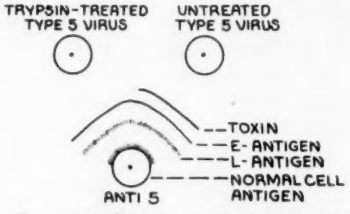

FIG. 5.—Diagrammatic representation of the effect of trypsin on the agar-diffusion pattern of crude type 5 adenovirus and suggested identities of precipitin lines.

free from toxin, the separation of toxin from contaminating material, in this case the L antigen, was not accomplished. Two observations, however, furnished a basis for separation and purification of the E antigen and toxin: (1) an E antigen-toxin mixture essentially free from L antigen and virus could be obtained from the chromatography of crude type 5 virus on DEAE at pH 7.2, and (2) E antigen was sharply separated from toxin by chromatographing E-T complex material on DEAE at pH 5.2. This method is exemplified in the following experiment. A pool of eluates containing a mixture of E antigen and toxin from a preliminary separation of type 5 antigens on a DEAE column at pH 7.2 was dialysed against 0.01*M* acetate buffer at pH 5.2. The dialysate was centrifuged to remove a light precipitate which formed during dialysis, and the clear supernatant fluid was added to a DEAE column at pH 5.2 as described in the previous section. Each eluate obtained from this column was titrated for toxin and for complement-fixing antigens employing various antisera.

The pattern of reaction obtained with type-specific rabbit antiserum revealed two peaks of complement-fixing activity (Fig. 7*a*). The second of these peaks corresponded with elution of toxin at the expected molarity of 0.16 *M* NaCl. Antisera to heterologous virus types, in this case anti-2 and anti-4, reacted only with the 0.16 *M* NaCl eluting material (Fig. 7*b*). These data support the concept that toxin is a common adenovirus antigen and as such is responsible for the lack of total specificity hitherto noted with the E-T complex (Figs. 2*b* and 2*c*). The CF

antigen recovered in the original effluent was devoid of toxic activity and reacted only with antisera produced in response to type 5 antigens (Figs. 7*a* and 7*c*). This material represented the E antigen and behaved in the manner expected of a type-specific antigen.

These experiments clearly indicate that type 5 adenovirus-infected cells produce in addition to infectious virus a group-specific antigen (the L antigen), a type-specific antigen (the E antigen), and a third soluble component possessing toxin-like properties which reacts as a second group-specific or common antigen.

The relationship of virus-induced antigens to normal host cell antigens: To ascertain whether the virus-induced antigens were contaminated with host cell antigens or, alternatively, were antigenically related to normal cell materials, each of the eluates from a chromatographic separation of type 5 antigens was tested for

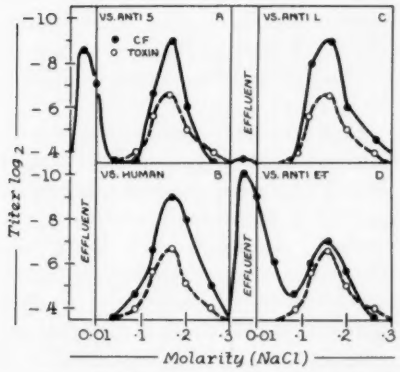

FIG 6.—Complement-fixation patterns revealed by the reaction of various antisera with eluates obtained from chromatography on DEAE of homogenates of type 5-infected HeLa cells at pH 5.2. The elution pattern of toxic activity is included for purposes of comparison.

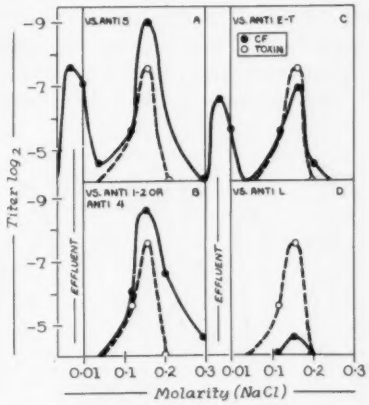

FIG. 7.—Separation of E antigen and toxin by chromatographing E-T complex material on DEAE at pH 5.2. Each eluate was tested with various antisera to reveal the nature of the CF antigens. E-T complex material was obtained from the chromatography at pH 7.2 of an homogenate of type 5 adenovirus–infected HeLa cells.

complement-fixation with antiserum directed against normal cell lysates (anti-NCE) as well as with antiserum specific for type 5 virus. The results of a typical experiment are shown in Figure 8. It is evident that the quantity of complement-fixing antigen in these eluates as measured with anti-NCE did not correspond with the elution patterns of virus-induced antigens. Only a single small peak of host-cell complement-fixing antigen was observed, eluting maximally at a molarity of 0.04 *M* NaCl. The slight contamination of E antigen with normal cell components was reduced below detectable quantities by a second chromatography of the E antigen peak.

Agar diffusion tests employing these same eluates revealed a single precipitin band shared by the 0.04 and 0.08 *M* NaCl eluates. This line, however, was not continuous with either of the precipitin bands characteristic of the E-T complex (Fig. 3).

These data were further verified by chromatographing normal cell homogenates at pH 7.2 under the same conditions used for homogenates of virus-infected cells. Each eluate was tested for normal cell antigens by employing agar diffusion as well as complement-fixation techniques. Antiserum to normal host cell materials (anti-NCE) was utilized in these tests. Both techniques indicated the presence of only a single peak which exhibited complement-fixing as well as precipitating activity. This antigen or antigens eluted maximally at a molarity of 0.04 *M* NaCl as anticipated from the preceding tests.

Virus-induced antigens in adenovirus-infected monkey kidney (MK) and Hep 2 cells: The antigens produced by type 5–infected HeLa cells were characteristic relative to their chromatographic and immunologic behavior. Despite the observation that these antigens do not appear to be related to normal host cell materials, it seemed pertinent to ascertain whether the production of these antigens was controlled by the type of cell infected. In order to accomplish this aim, two questions were asked: first, do other cell lines infected with type 5 adenovirus synthesize antigens identical to those produced in infected HeLa cells; and secondly, do such antigens, if produced, possess the same chromatographic characteristics as those obtained from type 5–infected HeLa cells?

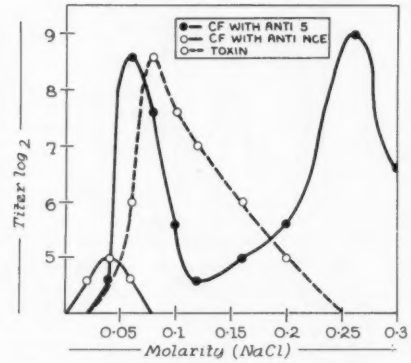

FIG. 8.—Comparison of the elution characteristics of host cell antigens and virus-induced antigens. Eluates obtained from the chromatography at pH 7.2 of type 5 adenovirus-infected HeLa cell homogenate were tested as indicated above.

Crude cell homogenates from type 5–infected monkey kidney (MK)[32] and Hep 2 cells were chromatographed at pH 7.2 in the manner previously described. As before, each eluate was titrated for complement-fixing antigens, toxin, and infectious virus. Elution patterns so obtained were identical with those noted with homogenates of type 5–infected HeLa cells (Fig. 1*a*). No immunologic differences between the E, L, and toxin antigens of type 5 virus produced in MK, Hep 2, or HeLa cells could be detected by complement-fixation or agar-gel diffusion techniques. Type-specific rabbit antiserum and antisera specific for each antigen were employed in these experiments.

It may be deduced from these data that insofar as the metabolic competence of the infected cell permits, the physical and immunologic characteristics of the soluble adenovirus antigens are rigidly determined by the virus genome rather than by the type of host cell. For this reason it would appear that the synthesis of these antigens is specifically associated with virus propagation.

Comparison of the chromatographic behavior of type 4 and type 5 virus preparations: It has been suggested that the lower-numbered adenoviruses may be divided into two subgroups, on the basis of cytologic changes induced by these viruses and the kinetics of neutralization and multiplication of the infectious agents.[16] On this basis, types 1, 2, 5, and 6 comprise one group and types 3, 4, and 7 the other. If

the differences between these groups were sufficiently marked, it might be predicted that suspensions of the latter group (types 3, 4, and 7) would exhibit similar chromatographic characteristics, which were, however, different from those of the agents of the first group. In this regard, it is pertinent to note that the results of Klemperer and Pereira indicate that the elution characteristics of types 2 and 5 virus on DEAE were identical.[13]

To test this hypothesis, homogenates of types 4 and 5 adenovirus–infected HeLa cells were chromatographed at pH 7.2 under identical conditions. Appropriate homotypic rabbit antisera as well as human convalescent serum were used to test duplicate samples of each eluate for complement-fixing antigens. The results of these experiments are shown in Figure 9. Unlike type 5 virus, homogenates of

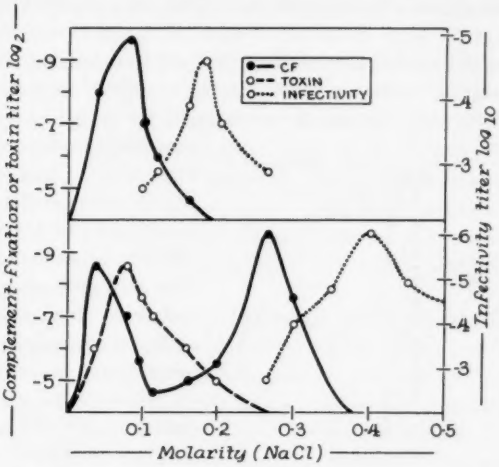

FIG. 9.—Comparison of the chromatographic behavior of homogenates obtained from type 4 and type 5 adenovirus-infected HeLa cells (upper figure, type 4; lower figure, type 5). The appropriate homotypic rabbit antiserum was used in each case to test for complement-fixation.

type 4–infected cells exhibited only a single peak of CF activity (Fig. 9) which correspond roughly with the E antigen of type 5 virus as regards its elution characteristics (Figs. 2*a* and 9). Even more striking was the difference in the chromatographic behavior of type 4 infectious particles, which eluted maximally at 0.16 *M* NaCl, and the type 5 agent which was recovered maximally in the 0.4 *M* NaCl eluates. Because type 4–infected HeLa cells produce only small amounts of toxin, it could not be detected in eluates obtained by the chromatographic procedure employed.

In order to gain some insight into the immunologic composition of the single CF peak, each of the eluates obtained from the chromatography of crude type 4 virus was tested for complement-fixing antigens employing antisera to heterologous virus types (anti-5 and anti-2) and antigens (anti-L and anti-E-T). With the exception of anti-E-T, each of these antisera reacted strongly with the 0.08 *M* NaCl eluates to reveal a pattern identical to that obtained with type-specific

rabbit antiserum as shown in Figure 9. These eluates comprising the type 4 CF peak may therefore be considered to contain a common or group-specific antigen immunologically similar to the L antigen of type 5 virus–infected cells. Antiserum prepared in response to the E-T complex of type 5 virus (anti-E-T) reacted very weakly with the material of the CF peak, a finding which was not surprising since the T antigen has been shown to be group-specific. Although one may consider from this evidence that the single peak obtained by chromatography of type 4 adenovirus was homogenous and a group antigen, other studies have demonstrated that this agent can be identified by complement-fixation with type 4–immune rabbit serum.[29, 30] It is probable, therefore, that the single peak of antigenic material contains type-specific antigen as well as the common adenovirus group antigen.

Chromatography of type 4 virus at pH 5.2: The success achieved in dissection of the antigens of type 5 virus by means of chromatography under acidic conditions suggested that similar procedures might be utilized to determine whether the single type 4 virus complement-fixation peak (Fig. 9) contained more than one antigen. Figure 10 illustrates the reaction of various antisera with eluates obtained when chromatographic separation of crude type 4 virus was carried out at pH 5.2. Under acidic conditions the single peak of CF activity seen at pH 7.2 (Fig. 9) was resolved into two well defined complement-fixing antigens (Fig. 10*a*). The use of antisera specific for heterologous virus types indicated that the first of these peaks was a common or group-specific CF antigen (Figs. 10*b* and 10*c*). This interpretation was substantiated by results obtained when antiserum specific for the common antigen (L antigen) from type 5 virus–infected cells was employed (Fig. 10*d*). In contrast, eluates comprising the second peak of complement-fixing activity eluting maximally at a molarity of 0.12 *M* NaCl, reacted only with antiserum directed against type 4 virus (Fig. 10*a*). These results indicate that like type 5 virus–infected cells, those infected with the type 4 agent produced a specific as well as a common CF antigen.

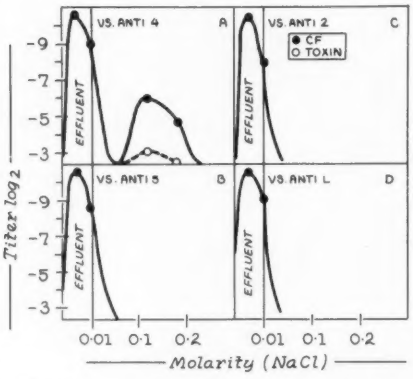

FIG. 10—Complement-fixing patterns obtained following chromatography of crude homogenates from type 4–infected HeLa cells at pH 5.2. Aliquots from each of the eluates were tested for complement-fixation with the various antiserum preparations shown above.

Discussion.—It is clear from the preceding data that a simple yet efficient separation of adenovirus-induced antigens may be effected by chromatographic procedures. By extending these methods, it was possible to obtain relatively pure preparations of the various soluble antigens associated with adenovirus-infected cells. In terms of quantitative recovery following a single cycle of chromatography, infectivity ranged from 30 to 80 per cent of original values, toxic activity from 80 to 100 per cent, whereas recovery of the complement-fixing antigens was consistently less than 30 per cent of the original amount.

When one combines the results of the experiments just described with those reported by Klemperer and Pereira,[13] it appears likely that in addition to virus, the lower-numbered adenoviruses characteristically elicit the production of three soluble antigens in infected cells. Two of these antigens were shown to be group-specific or common adenovirus antigens while the third was type-specific. The separation of these materials, either by chromatography,[12, 13] or by immunoelectrophoretic techniques,[33, 34] indicated that these antigens differed in certain physical characteristics. It is important to emphasize that similar antigens from types 4 and 5 virus–infected cells differed widely in their characteristics of elution from DEAE. These differences in chromatographic properties were unexpected in the case of the common or group complement-fixing antigens of these agents, since by immunological criteria these materials appeared to be closely related if not identical. Types 4 and 5 infectious virus particles also exhibited considerable differences in their elution optima.

Examination of the chromatographic characteristics of adenovirus antigens indicate two major patterns of behavior, one typified by the antigens of adenovirus types 2 and 5,[13, 14] the other by the antigens of type 4 virus. These data furnish an additional criterion to indicate that it may be possible to divide the lower-numbered adenoviruses into two sub-groups: adenovirus types 1, 2, 5, and 6 and virus types 3, 4, and 7. This evidence is compatible with the previously suggested division of the lower-numbered adenoviruses into two sub-groups on the basis of multiplication kinetics, neutralization by antiserum, and cytological changes induced by these agents.[16]

In several instances, a relationship has been shown to exist between soluble virus-induced antigens and certain components of the virus particle proper.[6, 7, 35] These data may be interpreted to indicate that the antigens in question were virus precursor materials or, less likely, degradation products originating from newly synthesized virus. At present there is no precise information regarding the relationship of virus to soluble antigens in the adenovirus-host cell system. There is, however, a certain amount of indirect evidence which serves as a basis for speculation about the nature of these antigens. When the relationship between adenovirus-induced antigens and the host-cell was examined, two findings were prominent: (1) adenovirus-induced antigens could not be shown to be related immunologically to normal host cell antigens, and (2) the immunological and physical nature of the soluble antigens and the infectious virus particles was determined solely by the type of infecting virus rather than by the origin of the host cell. These findings imply that the soluble adenovirus antigens are not merely products of host cell injury but rather are very specifically associated with the viral synthetic processes. It is possible, therefore, that at least certain of these antigens may be virus components existing either as a pool of precursor materials or as breakdown products originating from newly synthesized virus particles. When the acridine dye, proflavine, was added in the proper concentration to cultures of infected cells, it was possible to cause a 70 to 80 per cent reduction in the synthesis of infectious virus with no significant decrease in titer or CF antigens.[35] These data are consistent with the concept that toxin and CF antigens are not derived by the degradation of newly synthesized virus. This hypothesis is supported by evidence which indicates that the primary effect of proflavine is not on the synthesis of viral com-

ponents or precursors but rather is on the maturation or assembly of these materials into infectious virus.[36-39]

A picture of the assembly of virus components may be obtained from a consideration of the studies of Horne *et al.*[40] who concluded that the type 5 virus particle is an icosahedron with a shell constructed from 252 subunits. The diameter of a sub-unit is approximately 70 Å so that each may therefore represent a single protein molecule. These investigators suggested that the icosahedral structure could be obtained if three chemically different sub-units were employed to build the outer shell. In view of these findings, it is important to reiterate that type 5 adenovirus–infected cells characteristically produce three soluble antigens, which are postulated to represent virus precursor materials. The concept of virus maturation proceeding as an assembly of sub-units[6, 41, 42] leads to the possibility that two complement-fixing antigens and toxin combine with specific DNA to produce mature infectious adenovirus particles. In this regard, it is notable that adenovirus-infected cells produce a unique DNA[43] in addition to complement-fixing antigens and toxin. There is some evidence to indicate that this DNA represents precursor virus DNA.[44] It may be that the synthesis of excess viral DNA and protein, which appears to be characteristic for phage synthesis,[43-45] may also be common for multiplication of animal viruses in host cells.

Summary.—A chromatographic method for the separation and isolation of adenovirus-induced antigens from homogenates of infected cells is described. This procedure, in conjunction with immunological techniques, has been employed to identify, characterize, and compare the soluble antigens of types 4 and 5 adenovirus–infected HeLa cells. By such means it was shown that adenovirus-infected cells of various origins produce three soluble antigens in addition to infectious virus. These include a highly type-specific complement-fixing antigen, a group-specific or common complement-fixing antigen, and a toxin-like material which, when added to normal cells, causes rapid degenerative changes. The immunological behavior of the toxin is that of a group-specific or common adenovirus antigen. It was further shown that similar antigens from types 4 and 5 adenovirus–infected HeLa cells exhibited strikingly different chromatographic characteristics.

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SIGNIFICANT LIQUID STRUCTURES, VI. THE VACANCY THEORY OF LIQUIDS

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The liquid state is stable in a temperature range intermediate between that of solid and vapor. Usually, the liquid density is also intermediate. Exceptional substances such as water involve a structural shrinkage superposed on the usual expansion of the solid to liquid transition. It is natural to seek an explanation of the intermediate liquid state as an intimate mixture of solid and vapor. All the imperfections of the solid state should be even more abundant in the liquid. However, those imperfections which yield a large ratio of entropy increment to enthalpy increment will be correspondingly more abundant in the liquid and will be the significant structures in a quantitative theory.

The Liquid Model.—Holes of molecular size are assumed overwhelmingly abundant because (*a*) they confer gas-like properties on a neighboring molecule jumping into the hole and (*b*) a solid-like molecule obtains a positional degeneracy equal to the number of neighboring vacancies. Neglecting increase in volume due to holes of other than molecular size, the number of holes per mole of molecules is $(V - V_s)/V_s$, where V and V_s are the molal volumes of liquid and solid, respectively. The chance that a vacancy confers gas-like properties on a neighboring molecule is assumed proportional to the fraction of neighboring positions populated by molecules. This fraction is V_s/V if molecules and vacancies are randomly distributed. Thus, for random distribution of vacancies, the mole fraction of gas-like molecules is $(V_s/V)\ (V - V_s)/V_s \equiv (V - V_s)/V$. The remaining mole

fraction, V_s/V, of molecules are solid-like. The specific heat at constant volume for a liquid such as argon should thus be well represented by the equation

$$C_v = \frac{V_s}{V} 6 + \frac{(V - V_s)}{V} 3. \tag{1}$$

This is seen to be the case in Figure 1. This estimate of the number of solid-like and gas-like molecules was initiated by Walter and Eyring[1] in 1941.

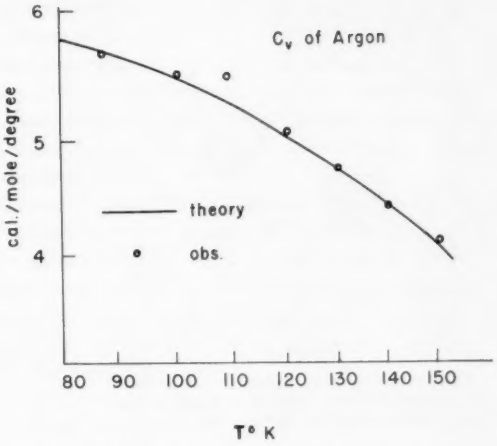

FIG. 1.—Specific heat of argon at constant volume plotted as a function of temperature. Circles represent observed values. The curve is a plot of equation (1).

The average number of vacancies next to a molecule if vacancies are randomly distributed is then

$$\frac{Z(V - V_s)}{V} = \left(\frac{ZV_s}{V}\right)\frac{V - V_s}{V_s} \equiv \frac{n(V - V_s)}{V_s}. \tag{2}$$

Here Z is the number of nearest neighbors and $(V - V_s)/V$ is the fraction of the neighboring positions occupied by vacancies. Since most simple liquids approximate a close-packed structure at the melting point, Z should have the value 12 and V_s/V is about 0.9 so that one expects $n \simeq 10.8$, which is the value which best fits the thermodynamic data for argon. It is natural to suppose that of the possible positions available to a molecule some one is energetically favored over the others by the way the neighbors are organized about it. This difference in energy between the best position and the average for the neighboring available positions should be proportional to the energy of sublimation, E_s, ahd inversely proportional to n_h, the number of holes; i.e. it should have the value $aE_s/n_h = aE_sV_s/(V - V_s)$. Figure 2 illustrates the energetic situation for a solid-like molecule comparing the most favored position with a neighboring vacancy.

It is now possible to write down the partition function, f, for a mole of rare gas such as argon

$$f = \left\{\frac{e^{E_s/RT}}{(1 - e^{-\theta/T})^3}\left(1 + n\,\frac{V - V_s}{V_s}\,e^{-\frac{aE_sV_s}{(V-V_s)RT}}\right)\right\}^{\frac{NV_s}{V}} \times \left\{\frac{(2\pi mkT)^{3/2}}{h^3}(V - V_s)\right\}^{\frac{N(V-V_s)}{V}} \left\{\left(\frac{N(V - V_s)}{V}\right)!\right\}^{-1}. \tag{3}$$

Using Stirling's approximation $X! = (X/e)^X$, this becomes

$$f = \left\{\frac{e^{E_s/RT}}{(1 - e^{-\theta/T})^3}\left(1 + n\,\frac{V - V_s}{V_s}e^{-\frac{aE_sV_s}{(V-V_s)RT}}\right)\right\}^{\frac{NV_s}{V}} \left\{\frac{(2\pi mkT)^{3/2}}{h^3}\,\frac{eV}{N}\right\}^{\frac{N(V-V_s)}{V}}. \tag{4}$$

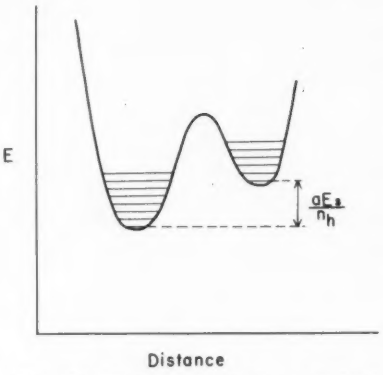

FIG. 2.—Potential curve for a molecule occupying the most favored position as compared with a neighboring vacancy.

Here N, θ, T, m, k, and h are Avogadro's number, the Einstein characteristic temperature of the solid, absolute temperature, mass of the molecule, Boltzmann's constant, and Planck's constant, respectively. The other quantities have been defined and the form of the partition function is determined by the model. The only constant not fixed by the model is a. The value of a required to fit the data is in accord with expectations that the energy for a molecule to move to a neighboring vacancy be small compared with the energy of sublimation, E_s.

In Table 1, the values obtained by Fuller, Ree, and Eyring[2] for the properties of argon using equation (4) are found. In this table, V, ΔS, P, and T represent molal volume in cc, the entropy of phase change, pressure in atmospheres, and temperature in degrees Kelvin. The subscripts m, b, and c indicate the melting point, boiling point, and critical point, respectively; $\Delta(\%)$ is the percentage deviation of the calculated from the observed values.

TABLE 1

PROPERTIES OF ARGON

	V_m, cc	ΔS_m, eu	P_m, atm	V_b, cc	ΔS_b, eu	T_b, °K	T_c, °K	V_c, cc	P_c, atm
Calc	28.90	3.263	0.732	29.33	19.04	87.29	149.7	83.68	52.93
Obs	28.03	3.35	0.674	28.69	17.85	87.29	150.66	75.26	48.00
Δ (%)	+3.11	−2.61	+8.55	+2.22	+6.68	0	−0.637	+11.2	+10.3

n = 10.80 a = 0.00534 E_s = 1888.6 cal/mol
V_s = 24.98 cc θ = 60.0°K

It should be stressed that our theory does not regard the liquid state as a mixture of solid and gas. A molecule has solid-like properties for the short time it vibrates about an equilibrium position, then it instantly transforms to gas-like behavior as it jumps into the neighboring vacancy. It is well known that nucleation of bubbles in boiling and crystal nucleation in freezing, if uncatalyzed, are very slow processes. These facts show that no solid or gaseous molecular arrangement as large as these nuclei exists in the liquid state.

Theory of Molten Salts.—The observed percentage change in volume of argon upon melting is 12 per cent. It is interesting to compare the theoretically expected volume change accompanying the melting of salts with the observed values as listed in Table 2.

Evidently the expansion of molten salts upon melting is about twice that of argon. This is to be expected from our theory. The entropy of melting comes from the

TABLE 2

VOLUME CHANGES AT THE MELTING POINTS

	NaCl	KCl	NaBr	KBr
Liquid volume (cc)	37.74	48.80	44.08	56.03
Solid volume (cc)	30.19	41.57	36.02	48.05
Per cent change	25.0	17.4	22.4	16.6

randomness introduced by vacancies. Since positive ions can occupy only half the vacancies while negative ions can occupy the other half, it should require twice the percentage expansion to get the same entropy increase for each kind of ion. This is in accord with the observations. The fact that only half the extra volume $(V - V_s)$ provides vacancies for each kind of ion means that n in the degeneracy term, $n(V - V_s)/V_s$, should be only about half as large for salts as for argon. This too is in accord with the findings, as one sees below.

The energy of vaporization for liquid argon (see equation (4)) falls off as $(E_sV_s)/V$, that is, inversely as the volume, as van der Waals suggested for gases long ago. This is not true for the molten salts since in one of the three dimensions, a Na^+ and Cl^- cling together and there is expansion only in the other two dimensions. Thus the energy should fall off more nearly inversely proportional to $V^{2/3}$.

Such considerations provide the justification for the following partition function used by Carlson, Eyring, and Ree[3] for molten salts.

$$f = \left\{\frac{\exp E_s/2RT(V/V_s)^{1/3}}{(1 - e^{-\theta/T})^3}\left(1 + n\,\frac{V - V_s}{V_s}\,e^{-\frac{aE_sV_s(V/V_s)^{1/3}}{(V-V_s)RT}}\right)\right\}^{\frac{2NV_s}{V}} \times \left\{\frac{(2\pi mkT)^{3/2}}{h^3}\,\frac{eV}{N}\,\frac{8\pi^2IkT}{h^2}\,\frac{1}{1 - e^{-h\nu/kT}}\right\}^{\frac{N(V-V_s)}{V}}. \quad (5)$$

The results found by Carlson *et al.*[3] for KCl are shown in Table 3. The properties in the gas-like part of the partition function are for the molecule of KCl.

TABLE 3

PROPERTIES OF FUSED KCl

	T_m, °K	V_m, cc	ΔS_m, eu	T_b, °K	V_b, cc	ΔS_b, eu	T_c, °K	V_c, cc	P_c, atm
Calc	1023	49.06	5.40	1684	71.20	21.63	3092	432	135.5
Obs	1049	48.80	5.8	1680	—	23.1	—	—	—
Δ (%)	−2.6	+0.53	−6.90	+0.24		−6.36			

$n = 6$ $a = 0.03000$ $E_s = 54.15$ kcal/mol
$V_s = 41.57$ cc $\theta = 170°$K $I = 2.195 \cdot 10^{-38}$ gm cm^2
$\omega = 305$ cm^{-1}

Our vacancy liquid theory applies with equal faithfulness to molten metals if we recognize that the necessary vacancies need only be large enough to accommodate the ion stripped of the valence electrons and these ions are only about a third the volume of the atom. That the viscosity data indicate that the ions in liquid metals maneuver as though they were independent of their valence electrons was pointed out long ago.[4] Thus, metals expand upon melting roughly one third as much as argon. Taking this into account by choosing values of n about three times as large as for argon and allowing for both atoms and diatomic molecules in the gas-like part of the partition function, the same faithful representation of thermodynamic and transport properties for the metals is obtained.[5] Again, our model seems to be powerfully supported.

Surface Tension.—The partition functions for liquids used in this and earlier papers of the series reduce to the solid partition function when $V_s = V$ and to the gaseous partition function for $V \gg V_s$. It is therefore to be expected that such a partition function will represent the surface layer if proper account is taken of the density gradient. For a simple closepacked liquid such as argon, it is supposed that layers will orient so that a molecule tends to have six nearest neighbors in the

same layer, three neighbors below and three above. Only E_s, the bulk energy of sublimation, should be appreciably different for a surface and a bulk molecule. The energy E_{s_i} of a molecule in the ith surface layer is then given by the equation

$$E_{s_i} = E_s \left(\frac{6}{12} \frac{\rho_i}{\rho_i} + \frac{3}{12} \frac{\rho_{i+1}}{\rho_i} + \frac{3}{12} \frac{\rho_{i-1}}{\rho_i} \right). \quad (6)$$

Here ρ_i is the density of the ith layer and the subscript increases with depth in the liquid. When i is the top liquid layer and $i - 1$ the first gas layer, one can write

$$\rho_{i-1}/\rho_i = \exp \left\{ -\frac{E_s}{2RT} \left(1 - \frac{T}{T_c} \right) \right\}. \quad (7)$$

This corresponds to a molecule in the top liquid layer possessing 6 nearest neighbors in the same plane, 3 in the neighboring liquid plane, but none in the gas layer at low temperatures. A molecule in the first gas layer, on the other hand, has only the three neighbors in the first liquid layer with none in the same gas layer nor in the gas layer above. Thus, the extra binding energy of a molecule in the first liquid layer over one in the first gas layer corresponds to six more neighbors out of a possible twelve or an extra binding energy of $E_s/2$. In keeping with this result, the exponent of equation (7) reduces to $E_s/(2RT)$ at low temperatures and vanishes at $T = T_c$, as it must since the densities become equal at the critical temperature.

One now proceeds to calculate the Gibbs free energy per mole, $G_i = A_i + pV_i$, by an iterative process until self-consistency is achieved. For the surface layer, one can first guess the density ρ_i and then set ρ_{i+1} provisionally equal to the density of bulk liquid.

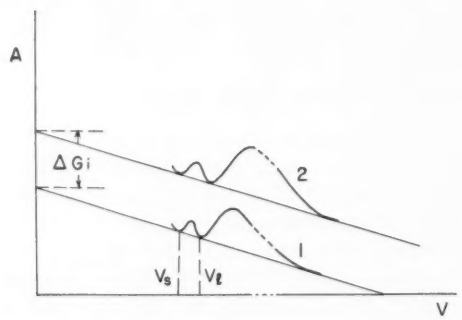

FIG. 3.—Schematic diagram of the Helmholtz free energy, A, plotted against volume at a fixed temperature for a bulk liquid (curve 1) and for a surface layer (curve 2). ΔG_i is the extra Gibbs free energy per mole of surface as compared with the bulk liquid.

Now after plotting A_i against V (cf. Fig. 3), the molal volume V_i of surface liquid can be obtained from the curve and is compared to V_i corresponding to the assumed value ρ_i. When, after repeated attempts, assumed and calculated values of V_i agree, one can recalculate ΔG_i for the layer i, etc. The whole procedure must continue until all assumed and calculated values of the density are self-consistent. The surface tension, γ, is then

$$\gamma = \sum_i \Delta G_i \frac{0.9165}{V_i} \left(\frac{V_s}{N} \right)^{1/3}. \quad (8)$$

The quantity $0.9165\ (V_s/N)^{1/3}$ is the volume of a layer of solid lattice one square centimeter in area.

In Table 4, observed and calculated values of the surface tension of argon are given following Chang, Eyring, Ree, and Matzner.[6]

In Table 4, the columns give the temperature Kelvin, molal volume of the top liquid layer, molal volume of the vapor, percentage of the surface tension calculated

TABLE 4

SURFACE TENSION OF ARGON (DYNE/CM)

$T°K$	V_l, cc	V_g, cc	1st, %	2nd, %	3rd, %	Calc	Obs	Δ, %
83.85	28.90	10,208	87.16	12.84	0	13.78	13.5	+2.09
85.5	29.08	8,493	86.45	13.55	0	13.43	13.1	+2.52
87.29	29.33	6,944	85.87	14.13	0	13.02	12.6	+3.33
90.0	29.80	5,246	85.35	14.65	0	12.22	11.9	+2.69

for the first, second, and third liquid layers, the calculated surface tension, the observed surface tension, and the percentage discrepancy. This is another striking success for the vacancy liquid theory. Values for other liquids are to be found in the paper of Chang *et al.*[6]

The calculated densities of successive layers in the neighborhood of the surface were calculated for argon and are plotted in Figure 4.

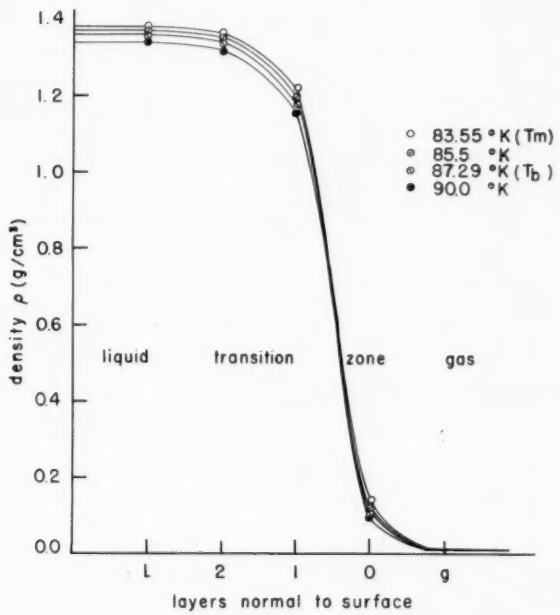

FIG. 4.—The density change in going from the liquid phase through the transition layers in the gas phase for argon at four temperatures.

Viscosity.—The vacancy theory of liquids has implicit in it a general theory of transport properties. Since there are solid-like and gas-like degrees of freedom in the liquid, both must be taken into account in calculating the viscosity. We follow the procedure outlined in the paper by Fuller, Ree, and Eyring (to be published). The shear plane lies between two molecular layers. If a fraction, x_s, of the shear plane is covered by solid-like molecules and the remaining fraction, x_g, by gas-like molecules, then the viscosity, η, which is the ratio of shear stress, f, to rate of strain, $\dot{s}$, is

$$\eta = \frac{f}{\dot{s}} = \frac{x_s f_s + x_g f_g}{\dot{s}} = x_s \eta_s + x_g \eta_g = \frac{V_s}{V}\eta_s + \frac{V - V_s}{V}\eta_g. \quad (9)$$

In accord with earlier procedure,[7] we now calculate the viscosity of solid-like molecules, η_s. We can write

$$\eta_s = \frac{f}{\dot{s}} = f/\left\{\sum_i \frac{\lambda \cos\theta_i k_i}{\lambda_1} \exp\frac{f\lambda_2\lambda_3\lambda \cos\theta_i}{2kT}\right\}$$

$$\simeq f/\left\{\frac{\lambda}{\lambda_1}\sum_i k_i\left(\cos\theta_i + \frac{f\lambda_2\lambda_3\lambda\cos^2\theta_i}{2kT}\right)\right\} \quad (10)$$

In Equation (10), $\dot{s}$ is the rate of shear corresponding to the velocity with which one molecular layer slips over the other divided by λ_1, the distance between layers (cf. Fig. 5). k_i is the frequency of jumping into the ith neighboring empty lattice site for $f = 0$. When f is not zero, the work helping the molecule move forward is the force $f\lambda_2\lambda_3$ multiplied by the component of the distance to the top of the barrier $(\lambda \cos\theta_i)/2$. Thus the rate of jumping into the ith site is $k_i \exp(f\lambda_2\lambda_3\lambda \cos\theta_i/2kT)$ and the corresponding distance jumped is $\lambda\cos\theta_i$, so that $\Sigma_i \lambda \cos\theta_i k_i \exp(f\lambda_2\lambda_3\lambda\cos\theta_i/2kT)$ is the velocity of the molecule due to all jumps into neighboring empty sites. Expanding the exponential gives the last form in equation (10). Since the sites are randomly distributed over the solid angle, the first term in the summation of equation (10) has the value $\Sigma_i k_i \cos\theta_i = 0$ and the second term

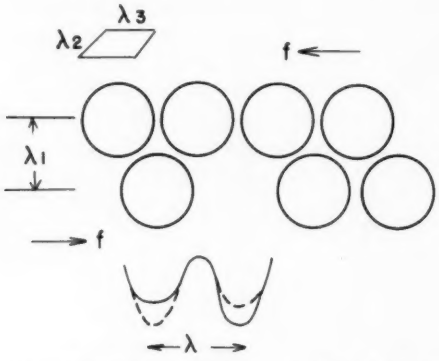

FIG. 5.—Distances between molecules in a liquid. λ is the distance between two successive equilibrium positions for viscous flow.

$$\sum_i k_i \frac{f\lambda_2\lambda_3\lambda}{2kT}\cos^2\theta_i = (\sum_i k_i)\frac{f\lambda_2\lambda_3\lambda}{6kT}. \quad (11)$$

Here we have used the fact that the average of $\cos^2\theta$ over space is $1/3$.

According to our vacancy liquid theory, a solid molecule can jump into all neighboring empty sites, so that

$$(\sum_i k_i) = \kappa \frac{kT}{h}\frac{n(V - V_s)}{V_s}(1 - e^{-\theta/T}) \exp -\frac{a'E_sV_s}{(V - V_s)RT}. \quad (12)$$

In equation (12), we have supposed the activation energy is $a'E_sV_s/(V - V_s)$ in accordance with Figure 2 and that the partition function for normal and activated states cancel out in the rate expression except for the reaction coordinate where the partition function for the normal state introduces the factor $(1 - e^{-\theta/T})$. Using Equations (11) and (12) to simplify (10) gives:

$$\eta_s = \frac{\lambda_1 h \exp\left\{a' E_s V_s/(V - V_s)RT\right\}}{(\lambda^2\lambda_2\lambda_3/6)\kappa(1 - e^{-\theta/T})n(V - V_s)/V_s}. \tag{13}$$

The term η_g is taken as the expression for gas viscosity

$$\eta_g = {}^1/_3 nm\bar{c}\lambda, \tag{14}$$

where n is the number of molecules per cc, m is the molecular mass, $\bar{c}$ is the average and λ is the mean free path. Using kinetic theory considerations, this becomes

$$\eta_g = \frac{2}{3\pi^{3/2}d^2}(mkT)^{1/2}, \tag{15}$$

where d is the molecular diameter. If we take $\lambda^2\lambda_2\lambda_3/\lambda_1 = V_s/N$, and substitute equations (13) and (15) into equation (9), the result is:

$$\eta = \frac{Nh}{V}\frac{1}{(1 - e^{-\theta/T})}\frac{6}{n\kappa}\frac{V_s}{V - V_s}\exp\frac{a' E_s V_s}{(V - V_s)RT} + \frac{V - V_s}{V}\frac{(2mkT)^{1/2}}{3\pi^{3/2}d^2}. \tag{16}$$

If we apply equation (16) to argon, we get the results shown in Table 5. The calculated values, η(calc) I, II, and III were obtained from equation (16) by assuming the following three sets of values of a' and κ, respectively: $a' = 0$, $\kappa = 0.42$; $a' = 0.01014$, $\kappa = 1$; and $a' = a = 0.00534$, $\kappa = 0.662$. One may see that the values of η (calc) I agree better than those of II and III. However, the value, $a' = 0$, is problematical, since the activation energy for flow, $a' E_s V_s/(V - V_s)$, should be equal or greater than the energy, $aE_s V_s/(V - V_s)$, which is necessary for a molecule occupying a neighboring vacancy (cf. Fig. 2). Thus, in the calculations of η (calc) III, a value of a' which equals a (= 0.00534) was assumed and κ (= 0.662) was determined so as to fit the viscosity at 84.25°K, whereas in the column of η (calc) II, κ was assumed to be unity and a' (= 0.01014) was determined. Both the η (calc) II and III decrease too rapidly. If one considers, however, that the temperatures in Table 5 range nearly from the melting to the critical temperatures and that the viscosities are at various vapor pressures from about 70,

TABLE 5

VISCOSITY OF ARGON IN MILLIPOISES

T, °K	V, cc	η(obs)*	η(calc)I†	η(calc)II†	(calc)III†
84.25	28.25‡	2.82	2.82	2.82	2.82
86.25	28.59‡	2.62	2.57	2.33	2.45
86.90	28.69‡	2.56	2.51	2.22	2.35
87.30	28.73‡	2.52	2.49	2.17	2.32
90.00	29.15	2.32	2.32	1.86	2.08
99.5	30.49	1.62	1.77	1.16	1.42
110.0	32.74	1.37	1.53	0.88	1.13
120	34.43	1.16	1.07	0.58	0.77
127	36.31	1.00	0.89	0.47	0.63
133.5	39.16	0.77	0.73	0.38	0.51
138.7	42.04	0.70	0.59	0.31	0.414
143	45.39	0.63	0.49	0.26	0.35
147	51.21	0.56	0.38	0.22	0.28
149	57.08	0.50	0.31	0.20	0.24

* Zhadanova, N. F., *Soviet Phys. J.E.T.P.*, **4**, 749 (1957).
† Calculated from equation (16), see the text.
‡ These values were obtained by interpolating the data from the *International Critical Tables*, ed. E. W. Washburn (New York: McGraw-Hill, 1926). For other volume data, refer to Zhadanova, *op. cit.*

to 48 atmospheres, it must be said that the agreement of η (calc) II and III with experiment is quite satisfactory. Our treatment of the viscosity of gas-like degrees of freedom as equal to the viscosity for a perfect gas can certainly be improved.

It is of interest to compare the above calculation of viscosity resulting in equation (16) with earlier procedures. Formerly the gas-like contribution to viscosity was neglected and the whole liquid treated as the relaxation of a lattice structure. This is a reasonable approximation near the melting point but becomes less appropriate with rise in temperature. The other improvement adopted here is the more explicit form that can now be written for the rate constant, k', for the stress relaxation. The fact that in the current expression the fluidity is proportional to the number of holes $n(V - V_s)/V_s$ provides justification for a somewhat analogous relation found by Batschinski.[8] It is especially satisfactory that our viscosity equation for the liquid passes over naturally into the gas equation. The present liquid theory also suggests that since $V - V_s$ becomes zero for the solid, the solid should become very viscous and further that nonequilibrium dislocations and vacancies must be chiefly responsible for the plasticity shown by solids. In earlier papers from this laboratory, a theory of the bulk viscosity of ordinary liquids[9] and of polymers[10] was developed. The authors used the earlier type of rate expression which did not explicitly include the factor, $(V - V_s)/V_s$, corresponding to the number of holes adjacent to a molecule. According to our present model of the liquid, the bulk viscosity should be the same as the shear viscosity except for a change in the meaning of the transmission coefficient, κ. For the bulk viscosity, κ, should express the chance that when a molecule jumps into a hole, the abandoned hole transforms into an elastically expanded lattice, a phonon, which then travels with the velocity of sound.

Any relaxation process involves a rate constant, $k' = \kappa(kT/h)\ (F^{\ddagger}/F_n) \exp\ (-\epsilon_0/kT)$, which has as one factor the ratio of the partition function for the activated state to the partition function for the normal state. If temperature or pressure brings a change in viscosity through modifying the partition function, F_n, for the normal state, the result is said to be due to a change in liquid structure. Such a structure change appears as a change also in the thermodynamic properties of the liquid. On the other hand, a similar change in viscosity may result from a change in the partition function, $F^{\ddagger} \exp\ (-\epsilon_0/kT)$, of the activated complex, and this is not reflected in the thermodynamic properties of the liquid. Thus, the development of the partition function of the liquid to explain thermodynamic properties is the necessary preliminary for deducing from viscosity the properties of the activated state. As a result of this study, we arrive at the picture of molecules of simple liquids shifting position chiefly by using vacancies and less frequently by making use of dislocations or fractional sized holes.

Diffusion.—A great deal of experimental evidence indicates that liquids subjected to stress relax by the same mechanism regardless of the nature of the stress. Thus, the activation energy for either ionic diffusion or conduction is ordinarily equal to the activation energy for viscous flow, indicating that the same elementary reactions are involved. Self-diffusion and viscous flow likewise show the same activation energy and therefore involve the same elementary process.

Consider the relationship between viscosity and diffusion. The diffusion coefficient is defined following Fick by the equation

$$J_i = -D_i \frac{dc_i}{dx} = u_i c_i. \tag{17}$$

Here J_i is the current density of matter of kind i. D_i, u_i, and c_i are the diffusion coefficient, velocity, and concentration of the ith kind of matter. In Figure 6, we have drawn a typical cross section of a condensed phase normal to the direction of flow. We suppose that molecule 7 diffuses upward by randomly jumping past the six articulating sectants 1 to 6. This is a necessary assumption, since the activation for shear mechanism is involved in diffusion. Now the rate of shear is

$$\dot{s} = u/\lambda_1, \tag{18}$$

where u is the forward velocity of molecule 7 with respect to its neighbors. Further,

$$\eta_i = f_i/\dot{s}, \tag{19}$$

where f_i, the shear stress, is the driving force on molecule 7 divided by the sum, A_i, of the six shear areas 1 to 6, that is, $A_i = 6\,\lambda_2\lambda_3$ or, for the most general packing, when instead of 6 there are ξ neighbors, $A_i = \xi\lambda_2\lambda_3$ (see Fig. 6). Equations (18) and (19) are the relations which have been used in deriving (10).

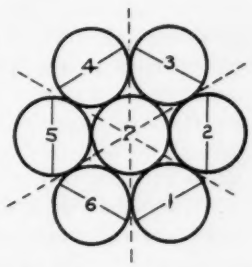

FIG. 6.—Hexagon normal to the direction of self-diffusion of molecule 7 in a close-packed liquid. Molecule 7 is diffusing forward.

Now for diffusion, the force on a molecule

$$F_i = -\frac{d\mu_i}{dx} = -\frac{d}{dx}(kT \ln a_i + \mu_{0i})$$

$$= -kT\frac{d \ln a_i}{dx} = -kT\left(\frac{d \ln a_i}{d \ln c_i}\right)\frac{1}{c_i}\frac{dc_i}{dx}. \tag{20}$$

Here μ_i, μ_{0i}, and a_i are the chemical potential, the chemical potential at unit concentration, and the activity a_i of the ith component. Also, we have

$$f_i = F_i/A_i = \frac{-kT}{\xi\lambda_2\lambda_3}\left(\frac{d \ln a_i}{d \ln c_i}\right)\frac{1}{c_i}\frac{dc_i}{dx} = \eta_i\dot{s}. \tag{21}$$

Combining equations (18), (19), and (21) with (17) yields

$$D_i = \frac{\lambda_1 kT}{\xi\lambda_2\lambda_3\eta_i}\frac{d \ln a_i}{d \ln c_i} \simeq \frac{kT}{\xi(V/N)^{1/3}\eta_i}\frac{d \ln a_i}{d \ln c_i}. \tag{22}$$

Equation (22) may be used to calculate the diffusion coefficient of argon at 84.12°K, here $a_i = c_i$. We take $\xi = 6$, $V = 24.98$ cc, and Zhadanova's value[11] for the viscosity, $\eta = 2.82 \times 10^{-3}$ poises. This gives $D = 1.98 \times 10^{-5}$ cm^2 sec^{-1} as compared with Corbett and Wang's observed value[12] $D = 2.07 \times 10^{-5}$ cm^2 sec^{-1}.

An alternative procedure in deriving equation (22) is to follow Einstein in equating the Stokes hydrodynamic viscous drag on a sphere,[13]

$$F_i = 6\pi r\eta u\frac{\beta r + 2\eta}{\beta r + 3\eta}, \tag{23}$$

to the thermodynamic drag, equation (20). The resulting equation is solved for u, which when introduced into equation (17) leads to

$$D = \frac{kT}{6\pi r\eta} \frac{d \ln a}{d \ln c} \left(\frac{\beta r + 2\eta}{\beta r + 3\eta}\right)^{-1}. \tag{24}$$

All the quantities except β, the coefficient of sliding friction, have been defined previously. β is the coefficient by which the relative velocity of two neighbors in contact must be multiplied to give force per unit area. If there is no slip at the interface, i.e. β is very large, the denominator in equation (24) is $6\pi r\eta$, while if $\beta = 0$, the denominator becomes $4\pi r\eta$. $4\pi r$ is the circumference of the circle drawn through the centers of the molecules 1 to 6, while $\xi(V/N)^{1/3}$, which plays the same role in equation (22), is the sum of the lengths of the straight lines which form the closed polygon joining the centers of the molecules 1 to 6. Equation (22) gives an excellent account not only of self-diffusion but also of mixed diffusion in uncomplicated cases.[14] The significant structure method with its recourse to absolute rate theory for treating relaxation problems has obvious advantages over hydrodynamics because of the limited number of models having tractable hydrodynamic solutions. This is exemplified by the calculation of the viscosity of a high polymeric system for which it is necessary to treat the random walk of a group of connected segments.[15] Onsager[16] has written an interesting review of theories of diffusion.

The significant structure model also gives a very satisfactory account for liquids of the coefficients of expansion, of compressibility, and therefore of C_p.[17] The thermal conductivity κ_l of liquids is represented rather well by an equation, $\kappa_l = \kappa_s V_s/V + \kappa_g(V - V_s)/V$, as shown by Walter Davis in a thesis in this laboratory. Here the subscripts l, s, and g on kappa indicate liquid, solid, and gas, respectively. That such a simplified model of the liquid state should represent thermodynamic and transport properties with the accuracy shown above is amazing. The significant structure method thus provides a new point of departure for treating all types of pure liquids and solutions.

Certain refinements of our theory are indicated:

1. Hole-hole interactions should be treated explicitly.
2. Dislocations, fractional-sized holes, stacking faults, and structures of the type discussed by Bernal[18] are sufficiently abundant to eliminate long-range order.
3. The long-range order which Debye finds in the critical region should appear as a consequence of a complete liquid theory.
4. Where there is pronounced structure change upon melting, as in the case of water, the values of E_s, V_s, and θ must be appropriately modified.

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A MATHEMATICAL AID IN OPTIMIZING ENGINEERING DESIGNS

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Introduction and Results.—An engineer is frequently faced with the problem of optimizing a design to obtain a minimization of total operating costs. The writer has found that in an important class of such problems the desired minimum can be found directly without recourse to the laborious procedure of first solving for the optimum value of the parameters and then substituting back into the cost equation or to the soulless operation of a machine which gives numerical answers but no insight. The purpose of this note is to present this direct solution.

In the class of problems to which the present technique is applicable, the operation cost C is expressed as a polynomial of the independent parameter. The technique is restricted, however, to the case where the polynomial contains one more term than the number of independent parameters. Thus, if we denote our parameters by $x_1, x_2, \ldots x_\sigma$, we are to minimize the cost

$$C(x_1, x_2, \ldots x_\sigma) = \sum_{i=1}^{n} T_i$$

where

$$T_i = a_i \prod_{j=1}^{\sigma} x_j^{\beta ij},$$

and where we have the restriction $n = \sigma + 1$.

The first step in our technique is to find a product of our n terms, each raised to an appropriate exponent α_i, which contains none of our σ parameters. This product we write as

$$\prod_{j=1}^{n} T_j^{\alpha j} = K$$

Such a set of α's is unique, apart from a common factor. In order to render the α's completely unique, we impose a normalization condition

$$\sum_{=1}^{n} \alpha_i = 1.$$

Our technique is not applicable to those cases in which normalization cannot be imposed, as when the sum of α's is identically zero. Usually the appropriate set of α's can be found by mere inspection. Thus, in the example

$$C(x, y) = ax + b/xy + cy^2,$$

we have $$(\alpha_1, \alpha_2, \alpha_3) = (2/5)(1, 1, 1/2),$$

and $$K = a^{2/5}b^{2/5}c^{1/5}.$$

In more obtuse cases, we obtain the n α's by solving the n linear equations

$$\sum_{j=1}^{n} \beta_{ij}\alpha_j = 0, \quad i = 1, 2, \ldots \sigma$$

$$\sum_{j=1}^{n} \alpha_j = 1.$$

Our second and final step is to write our desired minimum cost as

$$C_{\min} = K/\prod_{i=1}^{n} \alpha_i^{\alpha_i}.$$

Analysis.—The crux to our solution lies in adopting the appropriate viewpoint. Since our cost C has been formulated in terms of the σ parameters $x_1, x_2, \ldots x_\sigma$, our instinct is to regard these x's as the independent variables with which we must work. But we may with equal justification regard the n T's as our independent variables, independent apart from the condition that the product K remain constant. Adopting this second viewpoint we may state our cost problem as the minimization of the function

$$C(T_1, T_2, \ldots T_n) = \sum_{j=1}^{n} T_j$$

subject to the condition that

$$K(T_1, T_2, \ldots T_n) = \prod_{j=1}^{n} T_j^{\alpha_j}$$

remain a constant K.

Our cost problem, formulated in terms of the T's. is directly soluble by the undetermined multiplier[1] method of Lagrange. Thus Lagrange has shown that a constant λ (the undetermined multiplier) exists such that that set of T's which minimizes $C(T_1, T_2, \ldots T_n)$ subject to the condition that $K(T_1, T_2, \ldots T_n)$ remain a constant, also minimizes

$$C - \lambda K$$

for completely arbitrary variations in the T's. By direct differentiation, we readily show that this last expression has a minimum for that set of T's given by

$$T_j = \alpha_j \lambda K,$$

and hence obtain $$C_{\min} = \lambda K.$$

We may now determine the "undetermined multiplier" λ by substituting this

solution for the T's into the equation which defines K. We obtain

$$\lambda = 1/\prod_{j=1}^{n} \alpha_i^{\alpha_j}.$$

Comments.—If our minimum cost is sufficiently low to justify economically the design concept leading to the function $C(x_1, x_2, \ldots x_\sigma)$, we of course wish to find that set of parameters $x_1, x_2, \ldots x_\sigma$ which leads to this minimum cost. From our prior solution for the T's,

$$T_i = \alpha_i \lambda K,$$

we may frequently, by mere inspection, write the solution for the optimum values of the x's. When inspection is not sufficient, we resort to a solution of a set of linear equations developed by A. Fein.[2] This set of equations, as well as other interesting deductions following from the above-described technique, are described by Fein in a forthcoming paper.

2. When our solution for $C_{\min}$ is complex, as is the case where one of the α's is negative and fractional, the cost has been improperly formulated in terms of the parameters $x_1, x_2, \ldots x_\sigma$. Thus, suppose we are presented with the cost function

$$C = ax^{-2} - bxy + cy^2.$$

Our analysis gives at once

$$C_{\min} = \sqrt{-1}\, a^{1/2} b c^{-1/2}.$$

We conclude our cost was incorrectly formulated.

3. We have restricted ourselves to the particular case

$$n = \sigma + 1.$$

We shall now demonstrate that no minimum occurs when

$$n < \sigma + 1.$$

Suppose, for example, that

$$n = \sigma.$$

We then proceed by regarding one of the σ parameters $x_1, x_2 \ldots x_\sigma$ as a constant, only $\sigma - 1$ as variables. We then employ the technique developed in this paper to find the minimum $C_{\min}$ with respect to these $\sigma - 1$ variables. This minimum will contain as a factor that x_i, raised to some power, which was regarded as a constant. Such a factor cannot be minimized with respect to x_i, apart from the trivial value of zero when x_i occurs as an even power.

4. The case $n > \sigma + 1$ remains unsolved.

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*TRANSITIONS AND TRANSVERSIONS INDUCED BY DEPURINATING AGENTS**

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Communicated by M. R. Irwin, February 21, 1961

Mutations which are caused by single base pair changes in DNA have been divided into two different classes:[1] (1) transitions, where a purine is replaced by the other purine or a pyrimidine by the other pyrimidine, and (2) transversions, where a purine is replaced by a pyrimidine or vice versa.

The existence of transitions has been proved.[2, 3] For a series of mutants it has even been suggested which base pair had been changed.[3–5] But it was still an open question whether transversions really exist or whether for those mutations which have been shown to be non-transitions, a different mechanism is responsible.

By using depurinating agents like ethyl ethane sulfonate[4] and treatment with low pH,[6, 7] it seemed possible to induce both transitions and transversions. These agents preferentially remove guanine from DNA. Across the resulting gap not only cytosine can be incorporated into a new DNA strand producing the original standard-type, or thymine, giving rise to a transition, but also one of the two purines should occasionally be incorporated into the new DNA strand and cause a transversion. Thus, transversions containing a G-C pair at their mutant site should be inducible by these agents to revert to the original genome.

Material and Methods.—T_4 rII mutants and the techniques of growing and selecting them have been described by Benzer,[8] Benzer and Freese,[9] Freese[1, 2] and Brenner *et al.*[10]

The method of measuring reversion induction from rII to standard type is given in detail in the paper of Freese and Freese[3] for reversion induction by nitrous acid. The procedure for induction with ethyl ethane sulfonate has been described by Bautz and Freese[4] and that for hydroxylamine by Freese *et al.*[5] For reversion induction with AP and BD, spot tests have been described by Freese.[1]

The only method used in this paper which has not been described so far was the treatment of phages with low pH. T_4 wild-type phages were suspended in 0.5 *M* sodium acetate buffer, pH 4.2, and incubated at 37°C. After various times (between time 0 and 10 hours treatment), aliquots transferred into Tris-buffer (0.5 *M*) of pH 7.8 at room temperature.

Results.—Abbreviations: Adenine = A, Guanine = G, Thymine = T, Cytosine or 5-hydroxymethylcytosine = C, Ethyl ethane sulfonate = EES, Hydroxylamine = HA, Nitrous acid = NA, Bromodeoxyuridine = BD, 2-aminopurine = AP; Bromouracil-induced mutants = N mutants,[10] 2-aminopurine-induced mutants = AP mutants,[3] Proflavine-induced mutants = P mutants,[10] and EES-induced mutants = EES mutants.

(1) *Phage T_4 rII mutants induced by ethyl ethane sulfonate and low pH:* Phage T_4 wild type was treated with EES (0.11 *M*) for 30 min followed by incubation at 37°C for 24 hours in the presence of 1 per cent sodium thiosulfate. Under these conditions, the survival was about 10^{-2}; the mutation frequencies were about 12 times above the spontaneous background. Forty-five rII mutants were isolated and tested for their ability to revert spontaneously and for reversion induction by the two base analogs BD and AP.

The same tests had been performed previously on mutants induced by low pH

(pH 5, 45°C).[7] Mutants were isolated at an inactivation of 3×10^{-5} and the mutation frequency was about seven times above the spontaneous level. All EES-induced mutants did revert spontaneously. Out of 115 low pH-induced mutants tested, six did not revert, while all the others did revert and were mapped as point mutations. Table 1 shows that EES and low pH produce both transitions and nontransitions, the ratio being about 3:1.

TABLE 1

INDUCTION OF REVERSE MUTATIONS OF EES AND LOW pH INDUCED MUTANTS

rII Mutants induced by	No. of mutants tested	Base Analog		Per cent spontaneous background of non-inducible mutants
		Inducible (%)	Non-inducible (%)	
Ethyl ethane sulfonate	47	70	30	10
pH 5, 45°C[8]	115	77	23	15

(2) *Reversion induction of transitions:* It is known from chemical analysis that EES preferentially removes guanine from DNA.[4] Low pH treatment is known to depurinate DNA. The data of Tamm, Hodes, and Chargaff[6] indicate that at pH 1.6 guanine was removed first but after some time adenine reacted too. From these data it could not be concluded whether pH 4.2, under the conditions used in our experiment, preferentially removes guanine or removes it at the same rate as adenine.

In order to decide this question, a set of base analog induced mutants was tested for their reversion induction by low pH and the rate of induction was compared to the results obtained from experiments with EES.

As has been shown by Bautz and Freese,[4] EES induces the reversion of AP mutants. It does not induce or only very slightly induces the reversion of N mutants,[3] and some AP mutants. The same mutants were tested now for their reversion induction with pH 4.2. As can be seen from the data in Table 2, the same mutants that are highly inducible by EES to revert are also highly inducible by

TABLE 2

THE MUTAGENIC DIRECTION OF TRANSITIONS

Mutant	K_0/B_0 in 10^{-6}	α/β pH 4.2 in 10^{-6}	K_0/B_0 in 10^{-6}	α/β EES in 10^{-6}
AP 114	5.3	8.0	4.2	3.1
AP 275	1.7	7.6	1.07	1.5*
AP 72	1.29	5.2	0.76	1.0
AP 12	~0.5	~1.8	~0.85	~4.6*
AP 41	~0.3	~1.2	0.37	~2.3*
AP 156	0.007	0.01	0.04	0.08
AP 70	0.018	0.01	0.028	0.065*
N 29	0.013	0.01	0.04	0.03*
AP 83	0.011	$\lesssim$0.01	0.03	0.09
N 19	0.009	$\lesssim$0.01	0.07	0.016*
N 24	0.48	$\lesssim$0.08	0.32	$\lesssim$0.01*
AP 61	0.13	$\lesssim$0.03	0.18	$\lesssim$0.01*
N 31	0.028	$\lesssim$0.01	0.01	$\lesssim$0.01*
N 12	0.03	$\lesssim$0.01	0.03	$\lesssim$0.01*
N 7	0.016	$\lesssim$0.01	0.03	$\lesssim$0.01*
N 17	0.066	$\lesssim$0.01	0.02	$\lesssim$0.01*
r 320	0.013	$\lesssim$0.04	0.013	$\lesssim$0.02

The spontaneous reversion rates (K_0/B_0) and the absolute rates of reversion induction (α/β) per lethal hit are given for a number of transition mutants from reversion experiments with pH 4.2 and EES. $\alpha/\beta \lesssim$ means no detectable amount of induction.

* These data are from Bautz and Freese.[4]

treatment with pH 4.2. Actually, the separation of base analog induced mutants into the two possible classes (G-C → A-T and A-T → G-C) seems even clearer. The five AP mutants which can be highly induced to revert by both agents probably contain a G-C pair at their mutant site while the rest, N-mutants and also some AP mutants, are likely to have an A-T pair.

From these results, we conclude that under the conditions used, low pH preferentially removes guanine from DNA similar to EES. The data further suggest that the directional effect is even more pronounced.

(3) *Reversion induction of transversions:* Since EES and low pH remove guanine from DNA and thereby can induce transitions, it seems clear that they also should be able to induce transversions. Thus, EES and low pH can be used to prove the reversion induction of those transversion mutants which have a G-C pair at the mutant site. A mutant can be changed back to its original detectable standard type or into another rII mutant which cannot be recognized with our selective system.

Five EES mutants, 10 proflavine-induced mutants, and 15 spontaneous mutants were tested. By AP and BD spot tests and by reversion studies with nitrous acid, all these mutants were shown to be non-transitions. All of them did revert spontaneously, although with very different frequencies.

In Figure 1, the linear increase of revertants per viable phage with the number of lethal hits by treatment with EES and pH 4.2 is shown for EES-induced mutant

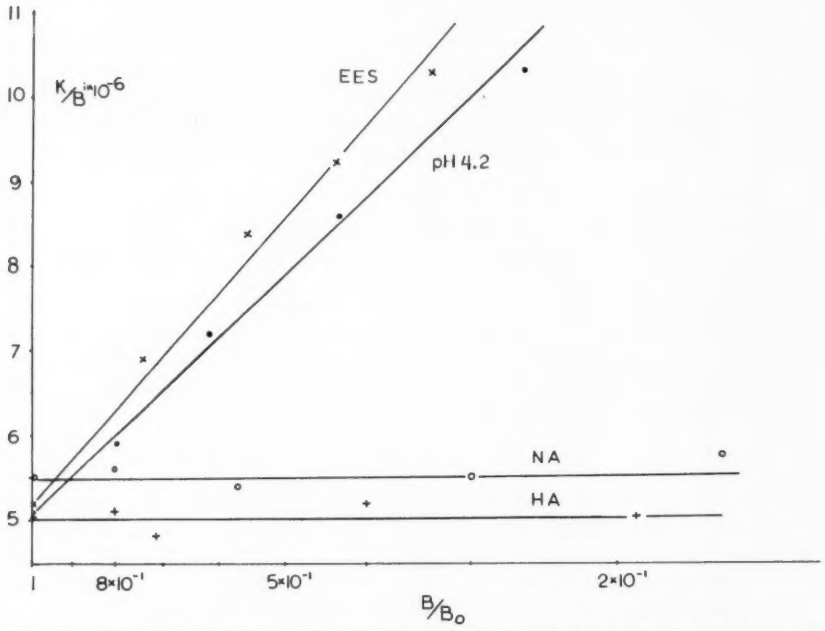

FIG. 1.—The values for the reversion induction of mutant EES 66 by EES and pH 4.2 show a linear increase over the spontaneous level with the number of lethal hits, i.e. (ln B/B_0) per viable phage. In contrast to this, there was no increase measurable after treatment with NA and HA.

TABLE 3

RATES OF REVERSION INDUCTION FOR NON-TRANSITION MUTANTS

Mutant	K_0/B_0	α/β pH 4.2	α/β EES	α/β NA	m.o.p.	AP	BD
r 111	160.0	66.0	70.0	<3	0.002	—	—
r 207	69.0	57.0	55.0	<2.5	0.001	—	—
r 132	197.0	55.0	50.0	<2	0.0005	—	—
r 157	175.0	40.0	50.0	<5	0.001	—	—
r 315	5.0	5.0	4.5	≲0.2	0.05	—	—
r 101	14.6	2.2	3.8	≲0.1	0.02	—	—
r 101	4.0	....	3.9	....	....		
r 105	6.1	2.4	3.5	≲0.1	0.02	—	—
r 179	6.6	2.4	3.4	≲0.05	0.02	—	—
EES 66	5.3	4.0	4.5	≲0.1	0.02	—	—
EES 116	7.8	2.9	3.3	≲0.1	0.03	—	—
P 11	~4.0	~1.3	≲0.8	≲0.2	0.01	—	—
r 131	0.4	≲0.1	≲0.1	≲0.1	0.05	—	—
r 114	0.7	≲0.2	≲0.1	≲0.1	0.05	—	—
r 117	0.6	≲0.1	≲0.1	≲0.01	0.1	—	—
r 194	0.5	≲0.1	≲0.1	≲0.01	0.05	—	—
r 178	0.02	≲0.01	≲0.01	≲0.01	0.2	—	—
r 123	0.02	≲0.01	≲0.01	....	0.2	—	—
r 289	0.1	≲0.01	≲0.01	≲0.01	0.1	—	—
EES 64	1.3	≲0.1	≲0.2	≲0.1	0.05	—	—
EES 122	1.1	≲0.3	≲0.2	....	0.05	—	—
EES 32	0.3	≲0.02	≲0.1	....	0.05	—	—
P 5	0.9	≲0.02	≲0.01	≲0.1	0.02	—	—
P 12	0.5	≲0.14	....	....	0.05	—	—
P 13	0.3	≲0.03	....	....	0.1	—	—
P 33	0.2	≲0.03	....	....	0.1	—	—
P 4	0.1	≲0.01	≲0.02	....	0.1	—	—
P 36	0.2	≲0.01	....	....	0.1	—	—
P 14	0.1	≲0.04	....	....	0.1	—	—
P 3	0.02	≲0.02	≲0.02	....	0.1	—	—
P 8	0.02	≲0.03	≲0.01	....	0.2	—	—

The spontaneous reversion rates (K_0/B_0) and the absolute rates of reversion induction (α/β) per lethal hit are given for a number of nontransition mutants from reversion experiments with pH 4.2, EES, and NA. $\alpha/\beta \lesssim$ means no detectable amount of induction was measured. m.o.p. (multiplicity of plating) = number of phages per bacterium plated. A dash in the AP and BD columns means that no revertants could be detected by spot tests.

No. 66. In contrast to the induction with EES and low pH, there was no increase over the spontaneous background after treatment with nitrous acid and hydroxylamine, both of which induce mutagenic transitions exclusively.[4, 6] From these results, it is concluded that mutant EES 66 was caused by a mutagenic transversion.

Of five EES mutants tested, two were reversion-inducible by EES and pH 4.2 and three were not, while out of ten proflavine-induced mutants, only one showed any effect. Of fifteen spontaneous mutants, eight were reversion-inducible and seven were not. The data are given in Table 3. Among the eight spontaneous mutants which could be induced to revert, four showed very high induction values and had high spontaneous reversion rates (these high spontaneous rates rendered the tests with nitrous acid less sensitive). The other inducible transversion mutants had lower rates of reversion induction and also lower spontaneous backgrounds, but the rate of spontaneous reversions was still considerably higher than for the non-inducible mutants.

We can think of two interpretations for these findings: (1) The highly reversion-inducible mutants are caused by transversions having now a G-C pair at their mutant site, while the less inducible ones are caused by transversions having an A-T pair. The mutants that are not reversion-inducible at all (but revert spontaneously) would then have to be of a different type, e.g. deletions of a base pair.

(2) Both the highly and the less reversion-inducible mutants are transversions containing a G-C pair, and at least many of the non-inducible ones are transversions containing an A-T pair. In case (2), the difference in the rate of inducibility would depend on the genetic site of the G-C pair and would be caused by a "higher mutagenic specificity."

We have some experimental evidence for the second interpretation. The highly reversion-inducible transversion mutants also have high spontaneous reversion rates and belong to genetic sites at which also spontaneous forward mutations occur especially frequently. In contrast, the less reversion-inducible mutants recur very rarely at the same site.

(4) *Difference in mutants which seem to belong to the same genetic site:* By locating the tested mutants on Benzer's genetic map of the rII region, the following was found: (1) Transitions and transversions can occur at the same genetic site as has been described earlier by Freese.[1] It has been shown for two transition mutants, by recombination tests, that each of them is located at the same genetic site as some transversion mutants. They were not reversion-inducible by EES and low pH and thus have an A-T pair at their mutant site. (2) Among the non-transitions of the same genetic site, we found mutants highly reversion-inducible by EES and pH 4.2 and non-inducible ones, corresponding to the high and low spontaneous reversion rates of these mutants (Table 4).[1, 9, 11] The highly inducible

TABLE 4

DIFFERENT MUTANTS AT THE "SAME GENETIC SITE"

Mutant	K_0/B_0	α/β pH 4.2	AP	BD
r 117	0.6	$\lesssim$0.05	—	—
r 132	197.0	55.0	—	—
N 17	0.07	$\lesssim$0.01	++	+
r 178	0.02	$\lesssim$0.01	—	—
r 207	69.0	57.0	—	—
r 320	0.01	$\lesssim$0.04	++	+
r 131	0.43	$\lesssim$0.06	—	—
r 157	175.0	40.0	—	—
?	....	....	..	..
r 114	2.7	$\lesssim$0.2	—	—
r 111	160.0	66.0	—	—
?	....	....	..	..

Two base analog non-inducible mutants available at each of the big spontaneous hot spots r 131, 114, and 117 showed very different spontaneous reversion rates (K_0/B_0) and also different α/β values, i.e. rates of reversion induction by pH 4.2. Mutant N 17, a base analog inducible mutant which had been located on the same genetic site as r 117 and r 132, was not inducible by pH 4.2 but showed strong reaction upon spot test with AP (++) and a weaker one with BD (+). r 178, r 207, and r 320 are three different mutants which seem to be at the same genetic site.

transversions are changes from a G-C pair back to the original C-G or T-A, whereas the non-inducible ones could be transversions with an A-T pair at the mutant site. Theoretically, these three kinds of mutants, the transition A-T, the transversion C-G, and a transversion T-A, could be derived from one base pair, G-C.

Summary.—(1) Low pH and EES induce either transitions or transversions.

(2) Both pH 4.2 and EES predominantly attack the G-C pairs; thus transitions mainly go from G-C to A-T and transversions from G-C to C-G or T-A.

(3) Transversions also exist among the spontaneous mutants.

I wish to thank E. Freese for helpful discussions and E. Bautz for the supply of EES.

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*FORMATION OF DUPLICATION-DEFICIENCY PRODUCTS BY ASYMMETRICAL EXCHANGE WITHIN A COMPLEX LOCUS OF DROSOPHILA MELANOGASTER**

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The possibility that pseudoalleles may represent closely linked genes having similar functions such that a position effect may exist between them, and the cytological evidence that such genes may occupy regions representing duplications, has led Lewis[1] to postulate that such cases may deal "with genes which were once (or are still) identical." The hypothesis states that before a gene with a new function can arise, there must first exist a pool of extra genes established through chromosomal duplication. One might expect to find pairing homologies within such a series of "old" and "new" genes in a manner comparable to such known duplications as Bar and Beadex-recessive, where unequal crossingover is a common feature. As yet, however, no clear case of other than strictly symmetrical pairing within complex loci has been demonstrated despite extensive studies of such loci in several organisms. Several examples of recovery of single products from a crossover event have been reported;[2-4] it is assumed in these cases that crossingover occurs in association with asymmetrical pairing and that the failure to recover one product is due either to its lethality or to a lack of distinctive phenotype necessary to distinguish it from one of the parental types.

Indication that asymmetrical pairing occurs within the white locus of *Drosophila melanogaster* first came from results obtained by MacKendrick,[5] who reported that w^{bl}/w^{aE} heterozygotes yielded both wild type and white recombinants, the latter through two different directions of crossingover. Judd[6, 7] also reported unexpected white crossover products from several heterozygous combinations of white mutants. On the assumption that the reciprocal product was not being recognized the author attempted to use attached-X chromosomes to recover both

products of a reciprocal exchange. This first attempt met with failure. Green[8] presented evidence that the white crossover class obtained from w^a/w^{a4} heterozygotes represented a cytologically undetectable loss of a portion of the white locus. This suggested that this class had its origin in crossingover within an asymmetrically paired portion of the locus. Green also failed, however, to find the product reciprocal to the deficiency type, and interpreted his findings as a putative nonreciprocal crossover event.

The present paper reports the recovery and partial analysis of reciprocal products resulting from crossingover within a nonhomologously paired portion of the white locus.

Recovery of Reciprocal Crossover Products.—To accomplish the recovery of exchange products known to be reciprocal, attached-X chromosomes were constructed after the method of Muller.[9] These chromosomes were made heterozygous at the white locus[10] (*w*, at 1.5 in the X chromosome) for w^a (apricot) and w^{bf} (buff) and carried the markers yellow (*y*, at 0.0; yellow body and bristles), yellow-2 (y^2; allelic and dominant to yellow, with yellow body and black bristles), scute (*sc*, 0.0+; missing scutellar bristles), zeste (*z*, $1^{\pm}$; brownish-yellow eyes), split (*spl*, 3.0; split bristles and rough eyes), echinus (*ec*, 5.5; enlarged eye facets). The constitution of the attached-X females is shown in Figure 1. The attached-X

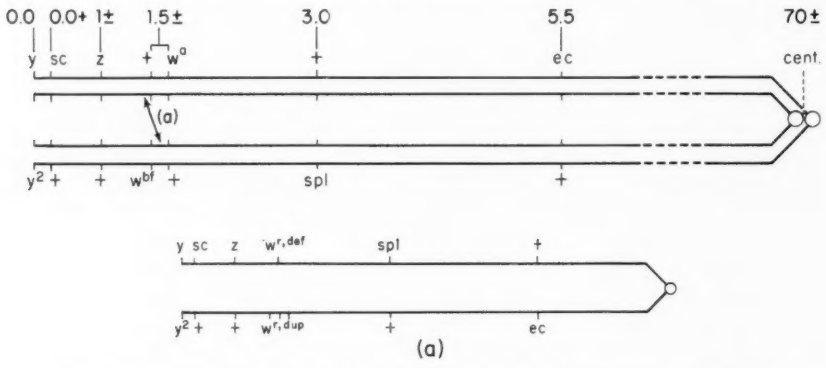

FIG. 1.—The upper figure is a diagram of the attached-X chromosomes used for detection of reciprocal products from an asymmetrical exchange. This asymmetrical exchange is indicated by (*a*) in this figure, with the resultant products diagrammed in the lower figure.

offspring from such a female are predominantly three types: (1) yellow-2 "dilute-apricot," which generally are the same genetic constitution as the parental female, (2) yellow scute "light-apricot" echinus (*y sc z* w^a *ec*), and (3) yellow-2 buff split (y^2 w^{bf} *spl*). Types 2 and 3 arise through certain types of exchanges between *ec* and the centromere, causing homozygosis for each group of linked mutants. These two rather frequent homozygous classes make it possible to determine the constitution of a single attached-X female simply by examination of her offspring.

Attached-X females of the constitution shown in Figure 1 were next made heterozygous for the second and third chromosome rearrangements, *SM1* and Ubx^{130}. These rearrangements increase the amount of crossingover in the *y-spl* region of the X chromosome approximately four times. These females were then mated

individually in food vials containing a standard yeast, cornmeal, molasses, agar medium, and the female offspring were examined for eye-color phenotypes which differed from those of the three predominant classes given above.

From approximately 31,000 female offspring, two yellow-2 white females were recovered in separate cultures. Examination of their sisters showed both had come from attached-X mothers of the type shown in Figure 1. These two exceptional females in turn produced offspring which allowed the determination of their genetic constitution. The two predominant homozygous classes produced were phenotypically (1) yellow scute white split and (2) yellow-2 "light-buff" echinus. Subsequent crossover studies showed the mutant zeste to be linked to yellow and scute. This linkage of *y*, *sc*, and *z* with *spl* and of y^2 with *ec* indicates that both females arose by a reciprocal exchange in the region between *z* and *spl*. Such a crossover is shown at point (*a*) in Figure 1. Evidence is presented below which indicates quite clearly that the yellow scute white split chromosome has a small, cytologically undetectable, deficiency for a part of the white locus and that the yellow-2 "light-buff" echinus product is the reciprocal duplication. These two products will henceforth be designated as $w^{r \cdot def}$ (white-recombinant, deficiency) and $w^{r \cdot dup}$ (white-recombinant, duplication). The "light-buff" phenotype of $w^{r \cdot dup}$ may be surprising in view of the fact that the phenotype of the heterozygote, $y\ sc\ z\ w^{r \cdot def}\ spl/y^2\ w^{r \cdot dup}\ ec$, is completely white. The phenotype of the heterozygote is due to an interesting interaction of $w^{r \cdot dup}$ with the mutant *z* on the homologous chromosome. The recognition and recovery of the reciprocal exchange products is based on this interaction, the significance of which will be discussed below.

Having determined that the exceptional types arose as a reciprocal exchange in or near the white locus, various tests were set up to determine the nature of the changes brought about by this event. The two crossover products first were detached from the attached-X chromosomes using $T\ (1;4)\ B^S$. Each of the types as hemizygous males proved to have the same phenotype as the corresponding homozygous females.

Analysis of Recombinant Chromosomes.—Males of each type were mated to Oregon-R females and the salivary gland chromosomes of the heterozygous female offspring were examined. Neither recombinant chromosome showed a detectable cytological change in structure. Both $w^{r \cdot def}$ and $w^{r \cdot dup}$ males were mated to *sp-w* females to test for a white locus deficiency. Green[8] showed that *sp-w/w-deficiency* females exhibit a phenotype inseparable from homozygous *sp-w* (eye light yellow with small reddish spots), while *sp-w* compounded with mutants of the white series located to the left of *sp-w* gives a uniform brown phenotype distinctly darker than either *sp-w* or the mutant with which it is compounded. Work in this laboratory confirms this test, and it appears to be a very reliable one for demonstrating white locus deficiencies. When females heterozygous for *sp-w* and the recombinant chromosomes were obtained, the $w^{r \cdot def}/sp\text{-}w$ females showed a phenotype identical with that of homozygous *sp-w*, while the $w^{r \cdot dup}/sp\text{-}w$ females showed a uniform brown eye color. This test clearly indicates that the $w^{r \cdot def}$ chromosome is deficient for some portion of the white locus. It is also clear from the method of recovery that the $w^{r \cdot dup}$ chromosome must be the reciprocal duplication.

Further evidence to support this hypothesis comes from the test to determine

the effect of $w^{r,\,def}$ and $w^{r,\,dup}$ on the mutant zeste. It has been shown by the work of Gans[11] that white deficiencies as well as some mutants of the white series act as dominant suppressors of *z*, i.e. females *z w*$^+$/*z w-deficiency* are wild-type in eye color. Green[12] showed that the white mutants which suppress zeste are those occupying the two rightmost recombination sites of the five sites now defined in the white locus,[13] i.e. those mutants apparently homoallelic to *sp-w* and w^{ch}. The heterozygote, $z\,w^{r,\,def}/z\,w^+$ is wild type in phenotype, showing that $w^{r,\,def}$ is a suppressor of *z*. Neither w^a nor w^{bf}, the two mutants carried in the parental chromosomes, act as suppressors. In this respect they are equivalent to w^+. On the other hand, the combination of $z^+\,w^{r,\,dup}/z\,w^+$ shows a slightly mottled, reddish-brown phenotype. This is similar to the phenotype described by Gans[11] for $z^+\,w^+/Dp\ (1;f)\ z^9/sc^{J4R}$, which essentially is $z^+\ w^+/z\ w^+/w^+$ (*z*, 1 dose; z^+, 1 dose; w^+, 3 doses). It is clear from this comparison that $w^{r,\,dup}$ is indeed a duplication for a portion of the white locus equal to 2 doses of w^+ with regard to its action on *z*. That $w^{r,\,dup}$ is acting as an enhancer of zeste is further shown by some combinations involving $w^{r,\,def}$, w^{11E4}, and w^1 (all are suppressors of zeste). Females $z^+\,w^{r,\,def}/z^+\,w^{r,\,dup}$ and $z^+\,w^1/z^+\,w^{r,\,dup}$ are light-buff in phenotype, while $z\,w^{r,\,def}/z^+\,w^{r,\,dup}$ and $z\,w^{11E4}/z^+\,w^{r,\,dup}$ females are white in phenotype. There can now be little doubt remaining that the reciprocal crossover products recovered from the described attached-X chromosomes are complementary deficiency and duplication products produced by a single exchange event.

The duplication and deficiency changes which occurred by recombination can be localized to the salivary gland chromosome doublet 3C2-3 by utilizing the white locus duplication $Dp\,w^{+51b7}$, described by Ratty.[14] This duplication which extends from 3C2-3 to 3D2 covers both $w^{r,\,def}$ and $w^{r,\,dup}$, i.e. $w^{r,\,def}/Dp\,w^{+51b7}$ and $w^{r,\,dup}/Dp\,w^{+51b7}$ males are wild-type in eye color. $Dp\,w^{+51b7}$ does not cover a deficiency of salivary gland chromosome band 3C1.

Recombinant Types Recovered from Free-X Experiments.—Considerable work has been done in this laboratory on recombination products recovered from non-attached-X w^a/w^{bf} heterozygotes. Three classes of crossovers are recognized from $y^2\,w^a\,spl\,ec/w^{bf}$ females. Phenotypically they are yellow-2 white and white split echinus, with the yellow-2 white being divided into two groups on the basis of their phenotype when compounded with *sp-w*. One of these appears to be a deficiency product strictly comparable to the *y sc z* $w^{r,\,def}$ *spl* described here. The other nondeficient yellow-2 white and the white split echinus classes from the free-X experiment have not yet been recovered from the attached-X experiment. The analysis of these products will be reported elsewhere when more is known concerning their origin and structure.

Exceptional white recombinants have also been recovered from w^a/w^{Bwx}, w^h/w^{Bwx}, w^{bf}/w^{Bwx}, w^h/w^e, and w^a/w^a females. These have all been tested against *sp-w* and all appear to be deficiencies by this test; all are male viable, however, and the salivary gland chromosomes are normal. The recombinants recovered from the first three heterozygotes listed above have also been tested for action on zeste. All act as zeste suppressors. Since the parental eye colors are quite different for each of the heterozygous combinations above, one can only assume that if the reciprocal duplication class is appearing, its phenotype must closely resemble one of the parental types in each case. If the results reported here may be extended

to these cases, one would predict that the reciprocal class does occur in each case and probably is similar to the lighter parental eye color in phenotype.

Discussion.—This demonstration of asymmetrical pairing and crossingover within the white region is of interest because of the information it gives to the study of chromosome organization and gene evolution. It is clear that pairing homologies exist between the subunits of this locus. This is most readily explained on the basis that these units were at one time (or still are) identical and arose through some mechanism leading to small chromosomal duplications. Such a process may account for the structural and functional complexity that has been demonstrated for the white locus.

If we visualize the five recombination sites now defined as points 1 2 3 4 5 along the chromosome, we can assign w^{bf} and w^{a} to points 2 and 3, respectively. It is not at all clear which of the sites are involved in the asymmetrical exchange, but a brief consideration of what is known about the functional subdivisions of the region may cast some light on this point. Green[12] has shown that with regard to the suppressor action on zeste, the mutants occupying the five sites fall into two groups. Sites 1, 2, and 3 contain nonsuppressor mutants while 4 and 5 contain zeste suppressors. The mutants in sites 1, 2, and 3 also show dosage compensation (i.e. hemizygous mutant ♂ exhibits as much or more pigment in the eye than homozygous ♀). With the exception of w^{h} which is located in site 4, all mutants occupying 4 and 5 fail to show dosage compensation. On this basis sites 1, 2, and 3 seem functionally distinct from 4 and 5. This is of interest when one considers the two exchange products in these respects. The $w^{r \cdot def}$ product is a *z* suppressor; since it is white in phenotype no measure of dosage compensation can be made. The $w^{r \cdot dup}$ product on the other hand is essentially a zeste enhancer and it does show dosage compensation. These observations could be interpreted as attributable to a loss of all or part of the nonsuppressor portion (sites 1, 2, and 3) of the locus in $w^{r \cdot def}$ and the duplication of this portion in $w^{r \cdot dup}$. Such duplication-deficiency products could arise by crossingover within the white locus if pairing homology exists between any two of the nonidentical sites. For example, pairing may take place in the following way:

1 2 w^{a} 4 5 (mutant w^{a} assigned to site 3).

1 w^{bf} 3 4 5 (mutant w^{bf} assigned to site 2).

If a crossover occurs between sites 1 and 2 in the w^{a} bearing chromosome and between sites 2 and 3 in the w^{bf} bearing chromosome the resultant products would be 1–3 4 5 (deficient for site 2) and 1 w^{bf} 2 w^{a} 4 5 (duplicated for site 2). Asymmetrical pairing and crossingover may of course occur at other points, resulting in duplication-deficiency products for other sites as well. Some evidence for this comes from the report by Green[8] that the white recombinant deficiency obtained from w^{a}/w^{a4} (both apparently located in site 3) does not show a suppressor effect on *z*. This may mean that this product represents a deficiency for site 4 or 5 (or both). In any event the recombinant-deficiency product reported by Green is certainly different from those reported here with respect to suppressor action on the mutant zeste.

One other point can be made regarding the type of gene function in the white series. The white mutants, in general, are considered to be hypomorphs after

the system of Muller.[15] Green[8] reports that a tandem duplication for the whole w region which contains the mutants w^a and w^{a4} is distinctly more nearly normal than either mutant separately. However, when two pseudoalleles of the white series are coupled in the same chromosome the resultant phenotype is usually comparable to that of a deficiency for the white region (the exceptions to this rule are those combinations involving the mutant w^{Bwx} at site 1). These two observations are not compatible unless one invokes a position effect to account for the phenotype of the double mutant. In the case reported here, $w^{r,\,dup}$ is distinctly lighter than w^a and slightly lighter than w^{bf}. It is not equivalent to a white deficiency but it is less normal than either w^a or w^{bf}. It would seem that these observations are most readily explained on the basis that the recombinationally discrete units are *not* functionally discrete. The $w^{r,\,def}$ would then supply no gene product or an inactive one, while $w^{r,\,dup}$ would supply one which is less normal than from either w^a or w^{bf} but presumably more effective than one from $w^{bf}\,w^a$ coupled in a conventional manner in the same chromosome. Of course, one could again invoke a position effect to account for these phenotypes and thus maintain the view that the units defined by recombination are discrete functional units as well. The results presented here still do not allow a clear decision concerning this problem of what the recombination sites represent in terms of the functional genetic unit involved. One might view the hypothesized evolution by duplication process as involving the whole functional unit, or, just as logically, as involving only small segments of it. This point can be answered only when more is known concerning the structure and function(s) of the white region.

Summary.—A method for recovery of reciprocal products resulting from asymmetrical exchange within the white locus of *Drosophila melanogaster* is described. The analysis of the two complementary exchange products indicates they represent a duplication and a deficiency for a portion of the locus.

The significance of such an exchange is discussed with regard to gene evolution and with regard to the structure and function of this complex locus.

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*THE PROPERTIES OF ALTERED PROTEINS FROM MUTANTS BEARING ONE OR TWO LESIONS IN THE SAME GENE**

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The experimental examination of the relationship between gene structure and protein structure is now possible as a result of recent developments in genetics and protein chemistry. A number of studies on genetic fine structure, such as those of Benzer,[1, 2] have shown that it is possible to determine the order of mutationally altered sites within a single gene. Techniques for identifying and locating mutationally induced amino acid changes in a protein molecule have been developed by Ingram[3, 4] and applied in his elegant studies of normal and genetically altered forms of human hemoglobin.[3–5] These techniques are currently being employed in the study of several gene-protein systems and one of these systems, the A gene and the A protein of the tryptophan synthetase of *Escherichia coli*, is the subject of this report.

Escherichia coli tryptophan synthetase consists of two separable protein components designated A and B.[6, 7] Together, these proteins catalyze the following three reactions: (1) indole + L-serine → L-tryptophan, (2) indole glycerol phosphate ⇌ indole + triose phosphate, and (3) indole glycerol phosphate + L-serine → L-tryptophan + triose phosphate.[7] Reaction (3) is believed to be the physiologically important reaction in tryptophan biosynthesis.[7, 8]

A large number of strains have been isolated in which one or the other of these two proteins has been affected by mutation. Some of the A mutant strains produce an enzymatically altered protein that reacts with antibody to normal A protein, while other A mutant strains show no trace of any protein either enzymatically or immunologically related to normal A protein.[9] The B protein is normal in all of these A mutants. The same type of mutational effects are observed in B mutants.[9] The genetic regions governing the A and B proteins are located immediately adjacent to one another on the genetic map.[9]

The A protein of tryptophan synthetase has several characteristics that make it a favorable protein for studies of the gene-protein relationship. It can be obtained in large amounts—up to 2 per cent of the total extractable protein; it is a protein of moderate size, with a molecular weight of approximately 30,000; and it can be isolated and purified without difficulty.[10] Furthermore, some of the A mutants produce altered forms of the A protein which are particularly suited to quantitative studies for they participate in reaction (1) when mixed with normal B protein and they react with antibody to normal A protein.

The experiments presented in this paper show that altered A proteins produced by most of the mutants can be distinguished from the normal protein on the basis of their physical properties. It is further shown that the combined effects of separate mutational alterations within the A protein can be studied by examining the proteins produced by strains containing two different mutant sites within the A gene.

Materials and Methods.—Production and classification of mutants: The mutant

strains examined in this study were isolated by penicillin selection following ultraviolet irradiation of wild-type *E. coli* K-12.[11] Each strain presumably resulted from a separate occurrence of mutation within the A gene. Mutants with alterations in the A gene can grow on indole since the B protein alone can catalyze the conversion of indole to tryptophan. These A mutants accumulate indole glycerol and cannot grow on minimal medium or minimal medium supplemented with anthranilic acid.

Enzymatic procedures: Cultures used to prepare extracts were grown on a glucose-minimal medium[12] in the presence of levels of indole sufficient to allow growth to late log phase. It had previously been shown[9] that tryptophan auxotrophs form high levels of both the A and B proteins under these conditions. The cells were harvested by centrifugation, suspended in cold 0.1 *M* tris buffer at pH 7.8, and disrupted in a 10 KC Raytheon sonic oscillator. Particulate debris was removed by centrifugation. Virtually all of the A and B protein remains in the supernatant solution. Unless otherwise specified, the enzymatic assays reported in this paper were carried out with such extracts. Partially purified preparations, obtained as described previously,[13] were employed in some experiments.

The determination of A enzymatic activity involves measuring indole disappearance in the presence of pyridoxal phosphate, serine, and an excess of component B.[7] One unit of A activity corresponds to the conversion of 0.1 μ *M* of indole to tryptophan in 20 minutes at 37°C. Component B activity is measured in the same manner but in the presence of excess component A. Protein was determined by the method of Lowry *et al.*[14]

In the experiments reported in this paper the neutralization of the A activity in reaction (1) by antiserum to normal A protein was measured after incubation of extracts with antiserum for 10 minutes. Antibodies to component A inhibit the enzymatic activity of the A protein in reaction (1) and the degree of inhibition is proportional to antibody concentration to about 70 per cent of complete inhibition. One unit of antibody is defined as that amount which inhibits one unit of the enzymatic activity of A protein in reaction (1). The preparation of antiserum specific to the A protein was described previously.[9]

The effect of acidification on the A protein was determined with preparations treated with 0.05 *M* $MnCl_2$ to remove nucleic acids. These preparations were then taken to pH 4.0 with 1 *M* acetate buffer, pH 3.2, and centrifuged to remove the precipitated proteins. Enzymatic activity was measured at pH 7.8.

The thermolability of A activity was determined on extracts diluted with a 0.5 per cent solution of bovine serum albumin in 0.05 *M* phosphate buffer, pH 7.8, and heated in a constant temperature water bath. Aliquots removed at 0 time and at subsequent intervals were immediately cooled by addition to tubes containing cold 0.05 *M* tris buffer, pH 7.8. The enzymatic activity of all samples was measured after the final sample was removed.

Genetic procedures: The methods reported by Lennox[16] were used for the preparation of transducing lysates and for performing quantitative transductions. However, for spot test transductions the recipient cells were grown on the surface of nutrient agar plates supplemented with 2.5×10^{-3} *M* $CaCl_2$. These transductions were performed by placing a drop of the donor lysate on the surface of selectively supplemented minimal-glucose plates containing 2.5×10^{-3} *M* $CaCl_2$

and then suspending cells from the nutrient agar plates in the drop.

Results.—Previous studies with the genetic region governing the A protein of tryptophan synthetase have indicated that the genetic unit of function consists of a large number of recombinable mutational sites.[15] If the linear sequence of mutable sites in the gene were directly correlated with the amino acid sequence in the protein it determines, the proteins produced by mutants that can recombine with one another would not be identical for they would bear different alterations in the primary polypeptide chain of the protein. To test this assumption and, in addition, to provide more information about the effects of mutation on the A protein, the altered proteins produced by the different mutant strains have been examined to see if they can be distinguished by simple criteria.

Of the 57 mutant strains that have been examined 18 produce an altered A protein that is enzymatically active in reaction (1) in the presence of normal component B. The other strains do not exhibit A activity, nor do they form any protein that can react with antibody to normal A protein. When extracts of the strains that form altered A proteins were examined for the presence of low levels of activity in reaction (3), the conversion of indole glycerol phosphate to tryptophan, no activity was detected (less than 1 per cent of the activity of wild-type protein in this reaction). These strains were then examined for abnormalities in the level of A protein by determining the ratio of A and B activities. The A/B ratio in fresh extracts of strains that form normal A and B varies only between 1.0 and 2.0[9] and, therefore, this ratio can be used to detect abnormalities in the level of A protein. All of the A mutants, except mutant *A 1*, produce high levels of both A and B activity and have a normal A/B ratio (Table 1). Extracts of

TABLE 1

ENZYMATIC AND IMMUNOLOGICAL ACTIVITY OF EXTRACTS OF A MUTANTS

A protein source	Specific activity* A	Specific activity* B	Ratio A/B	Relative neutralization by antibody to normal A
Wild type	2.9	2.7	1.1	1.00†
A 1	3.2	6.3	0.51	0.76, 0.81
A 3	43	18	2.4	1.4, 0.95
A 11	39	20	1.9	1.2, 0.98
A 23	23	16	1.4	1.1
A 24	18	13	1.4	1.2, 0.91, 0.81, 0.95
A 26	33	16	2.2	1.3
A 27	27	18	1.5	1.2
A 28	24	13	1.8	1.0
A 33	44	26	1.7	1.3
A 34	46	27	1.7	1.2, 0.86
A 35	25	15	1.7	1.0
A 36	11	7.5	1.5	1.0
A 37	37	20	1.8	1.2
A 41	36	24	1.5	1.1
A 45	42	20	2.1	1.0
A 46	31	17	1.8	1.1, 0.91, 1.0
A 48	48	20	2.4	...
A 53	19	11	1.7	...
12 other A mutants‡	0.5–1.0	32–51	...	...

* Units per mg protein in reaction (1).
† Normalized to 1.00 and all other values related to this.
‡ These mutants do not form any protein immunologically related to normal A protein.
** The apparent A activity in these preparations can be attributed to the activity of B protein alone in reaction (1), which has been shown previously to be about 1 to 2% of the maximal activity of B protein in the presence of excess A protein under the conditions employed in these assays.

mutant *A 1* consistently contain low levels of A and B and only $^1/_2$ as much A activity as B activity. The mutant A proteins were also examined in immunological tests to determine if they were as susceptible as normal A protein to inhibition by specific antisera. It was found that approximately the same amount of A activity was neutralized in each case (Table 1). Therefore, it appears that most of these mutations did not profoundly alter the ability of the A protein formed to combine with antibody to normal A protein or to participate with normal B in reaction (1).

Precipitation of mutant proteins at pH 4.0: Normal A protein is stable to mild acidification and this characteristic has been used in the purification of the protein.[13] When extracts containing normal A protein are taken to pH 4.0 and the precipitated proteins removed by centrifugation, most of the A activity remains in the supernatant. When extracts of the various A mutants were subjected to this treatment, it was found that six (*A 11*, *A 26*, *A 37*, *A 41*, *A 45*, and *A 48*) lost approximately 90 per cent of their A activity (Table 2), while extracts of the other

TABLE 2

ACID SENSITIVITY OF MUTANT AND NORMAL A PROTEINS

A protein source	Per Cent Loss of A Activity* in the pH 4.0 Supernatant: First treatment	Second treatment
A 11	89	96 (99.6% of original)
A 26	87	96 (99.5% " ")
A 37	95	89 (99.4% " ")
A 41	93	90 (99.3% " ")
A 45	91	...
A 48	94	...
Other A mutants† and wild type	32–58	49–73 (69–81% ")

* Activity in reaction (1).
† All of these mutants formed an altered A protein.

A mutants behaved like wild-type preparations. The 10 per cent activity remaining in the supernatant of acid-treated extracts of the six sensitive mutants was not due to the presence of protein molecules that were resistant to acid, for when the acid treatment was repeated the remaining activity declined to the same extent as the original activity.

The nature of the loss of activity was examined further by determining whether the A activity in these strains was destroyed or merely precipitated by the acid. It was found that the increased loss of activity observed in the supernatant of

TABLE 3

PRECIPITATION OF ACID SENSITIVE A PROTEINS

Extract	Treatment		Original activity* remaining in solution (%)
A 26 extract → (acid sensitive)	pH 4.0	→not centrifuged	73
		→centrifuged	13
A 37 extract → (acid sensitive)	pH 4.0	→pH 7.8, centrifuged	69
		→pH 4.0, centrifuged	13
A 23 extract → (control)	pH 4.0	→pH 7.8, centrifuged	71
		→pH 4.0, centrifuged	75

* Activity in reaction (1).

acid sensitive extracts was due to precipitation without pronounced inactivation (Table 3). When the A activity of an acid-treated extract of an acid sensitive mutant was measured both before and after removing the precipitated proteins, the dramatic loss of activity appeared only if the precipitate was removed. Moreover, when acid-treated extracts of both an acid sensitive and an acid stable strain were divided into two fractions and one fraction was reneutralized before centrifugation, the greater loss of activity from acid sensitive extracts occurred only when the extracts were centrifuged at pH 4.0. Therefore, the loss of activity from extracts of acid sensitive mutants can be ascribed to the presence of altered A proteins that are acid precipitable.

Heat inactivation of A proteins: Inactivation by heat was also employed to distinguish different mutationally altered A proteins. The results of heat inactivation experiments are presented in Figure 1, which shows representative inactivation curves for normal A protein and for all of the altered A proteins examined. Normal A protein, the acid precipitable proteins (from mutants *A 11*, *A 26*, *A 37*, *A 41*, *A 45*, and *A 48*) and the altered protein from one other mutant (*A 34*) retain approximately 50 to 70 per cent activity after 30 minutes at 52°C. On the other hand, the proteins produced by seven of the mutants (*A 23*, *A 24*, *A 27*, *A 28*, *A 35*, *A 36*, and *A 53*) are considerably more labile than the normal protein. Only 5 to 15 per cent of the original activity remains after the heat treatment. Another mutant protein, from strain *A 1*, is also more labile than the normal, but appears to be intermediate between normal A and the proteins produced by mutants such as *A 23*. Three of the mutant proteins, those produced by mutants *A 3*, *A 33*, and *A 46*, are more stable than normal A protein. The A protein from *A 46* retains 80 per cent of the original activity; the proteins from *A 3* and *A 33* appear to be fully active after heating at 52°C for 30 minutes.

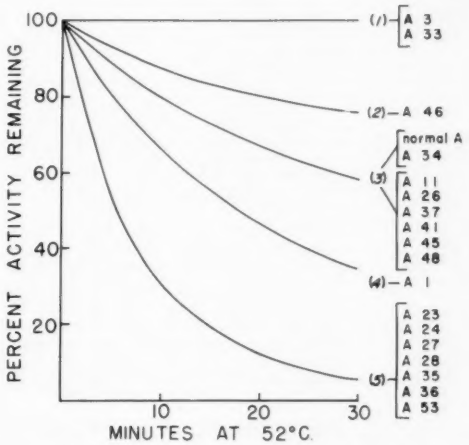

FIG. 1.—Heat inactivation of normal A protein and mutationally altered A proteins. The A activity of initial extracts was measured in reaction (1) in the presence of excess normal component B. These inactivation curves are representative of a large number of experiments. At least two separate extracts of each mutant strain have been tested.

Evidence indicating that the reaction to heat is a characteristic of the A proteins and not due to interaction with some other variable component in the extracts was obtained by testing the heat lability of partially purified preparations of A protein from several of the mutant strains. The partially purified preparations, which have no B activity, show the same heat stability as the initial extracts. Furthermore, studies designed to test the effect of B protein showed that it has only a slight effect on the heat inactivation of A protein. In another test, mixtures

of two A proteins with different stabilities were heated. The same results were obtained with mixtures employing either initial extracts or partially purified preparations. In Figure 2 the inactivation of A activity at 50°C in mixtures of partially purified preparations is compared with the inactivation of preparations

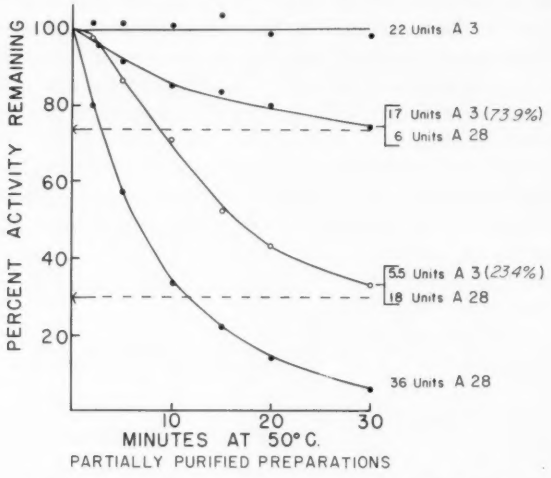

FIG. 2.—Heat inactivation of mixtures of heat labile and heat stable A proteins. Partially purified preparations from strains *A 3* (stable) and *A 28* (labile) were heated separately and in mixtures approximating 3:1 and 1:3 at 50°C. The dashed lines represent the expected proportions of A activity remaining and are equal to the total amount of stable protein added plus the residual amount of labile protein as calculated from the inactivation of the labile protein alone. A activity was measured in reaction (1) in the presence of excess normal component B.

of each protein type alone. If each protein were autonomous with regard to response to temperature, the inactivation curve should level off when most of the heat labile protein is inactivated and the total activity remaining at the end of the incubation period should be equal to the amount of the stable protein added plus that small fraction (approximately 10 per cent) of the labile protein that has not yet been inactivated. The observed results are in excellent agreement with the expectations. A similar experiment was performed at 60°C with partially purified preparations of normal A and a heat stable mutant A protein. These two proteins are readily distinguishable at the higher temperatures and appear to be autonomous with regard to their response to heating (Fig. 3).

Affinity for normal B protein: Partially purified preparations of A protein of each stability type were tested for ability to interact with normal B protein by measuring the amount of A activity required to permit $^1/_2$ of the maximal activity in reaction (1) of a limiting amount of B protein. The amount of A required for $^1/_2$ maximal activity of 1.0 unit of B protein was 0.53 units of normal A protein, 0.53 units of *A 11* protein, 0.56 units of *A 3* protein, 0.66 units of *A 46* protein, and 0.84 units of *A 28* protein. Although there appear to be minor differences in the ability of the various mutant A proteins to combine with B protein, the amount of mutant

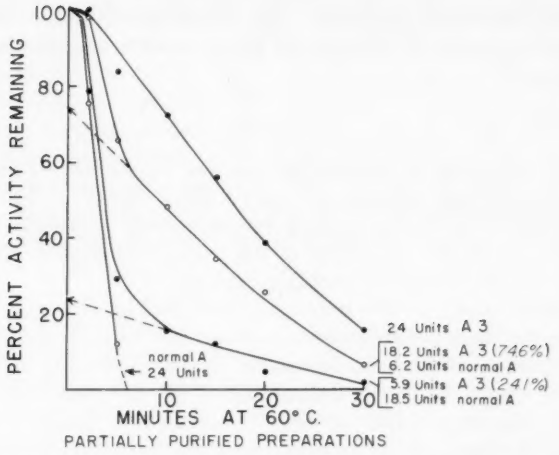

FIG. 3.—Heat inactivation of mixtures of a heat stable A protein and normal A protein. Partially purified preparations from strains *A 3* (stable) and from a strain producing normal A protein were heated separately and in mixtures approximating 3:1 and 1:3 at 60°C. The dashed lines are extrapolations of the final slopes of the inactivation curves and should intercept the ordinate at points approximating the proportion of the original A activity contributed by the more stable protein.

and normal A required is similar, indicating that these mutations had no great effect on the ability of component A to combine with component B.

Mapping: Mutationally altered sites within the A gene have been mapped by transduction and a map for some of these has been presented previously.[15] The relative location of the mutationally altered sites in the mutants studied here and some of the mutants previously studied is represented in Figure 4. All mutants that form altered proteins are clustered at the two ends of the A gene. Those mutants that do not form detectable altered proteins are scattered between these extremes.

The order of sites within the A region has so far been based on the frequency of recombination between two sites. In these tests the recipient bacteria contain one mutational alteration in the A region and, in addition, a requirement for histidine (*hist*) that is unlinked to the A region.[9] The transducing phage are grown on histidine-independent cells

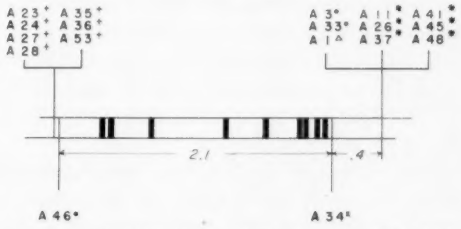

FIG. 4.—A partial map of the A gene. The thick bars correspond to the position of the altered sites in some of the A mutants that do not form proteins immunologically or enzymatically related to normal A protein. The thin lines correspond to the altered sites in mutants forming such altered A proteins. The physical properties of the proteins produced by these mutants are indicated by the symbols: + = heat labile, △ = heat labile, but more stable than the *A 23* group, ○ = heat stable, ● = heat stable, but less stable than *A 3* or *A 33*, * = acid precipitable, X = normal sensitivity to heat and acid. Since a different method for calculating recombination frequencies was used in this study[21] the map distances presented here are only ½ the distances reported previously.

that contain a different A mutation. The frequency of tryptophan-independent recombinants is expressed as a fraction of transduction to histidine independence.

recipient:	Ax	+	hist$^-$
donor:	+	Ay	hist$^+$

Since the ratio of transduction of the histidine and tryptophan regions is relatively constant and varies only between 0.4 and 0.6 for a variety of donor lysates and recipient cultures tested, this method for expressing recombination frequency automatically corrects for differences in the transducing titer of the lysates and differences in competence of the recipient cultures.

Mapping within clusters: The resolution of different genetic sites by the method described above is limited by reversion of recipient cells to tryptophan independence, which in some cases is frequent enough to preclude detection of recombination below 0.05 per cent. To permit examination of recombination below this value, a method has been devised that virtually eliminates reversion as a limiting factor in recombination experiments. In this method the recipient cells carry a cysteine marker (*cys*) linked to tryptophan in addition to the unlinked histidine marker. The donor bacteria again carry a different tryptophan mutation. Both the number of tryptophan-independent recombinants among the cysteine-independent trans-

recipient:	Ax	+	cys$^-$	hist$^-$
donor:	+	Ay	cys$^+$	hist$^+$

ductions and the total number of transductions to cysteine independence are determined. The frequency of recombination is expressed relative to the total number of transductions in the tryptophan region as calculated from the number of transductions to cysteine independence. Because of the loose linkage between cysteine and tryptophan, an appreciable fraction of the tryptophan-independent recombinants will also be transduced to cysteine independence regardless of the order of the two altered sites in the A gene. However, a recipient cell that has not been transduced will be unable to grow on histidine alone unless reversion of both the tryptophan and cysteine markers has occurred. Rare recombinants can be distinguished from contaminants by testing for the presence of the unlinked histidine requirement.

The clustered mutants that form altered proteins were tested for separability using this method. The results are presented in Table 4.[17] With the exception of *A 34* and *A 46* all of the clustered mutants appear to be inseparable. *A 34* recombines with the other mutants at the right end[18] of the A gene and *A 46* shows a very low frequency of recombination with at least some of the mutants located at the left end of the A gene. Figure 4 summarizes our present knowledge of the location of the mutated sites in mutants forming altered proteins and of the physical properties of the A proteins produced by these mutants. There is a striking correlation between map location and physical properties. The altered sites in all of the strains that form acid precipitable A proteins appear to be at the same site at the right end of the A genetic region. The genetic alterations in the two mutants that form heat stable proteins (*A 3* and *A 33*) also map at this site. The altered sites in the strains that form proteins less stable to heat than normal A

protein, with the exception of strain *A 1*, all map together at the left end of the A region. Mutant *A 1* maps at the opposite end of the gene and appears to be inseparable from the site in mutants forming heat stable or acid precipitable A proteins. The alteration in *A 34*, a strain which forms an A protein with normal heat and acid stability, is located approximately 0.4 units from the site of the mutations giving rise to acid precipitability and heat stability. *A 46*, a strain that makes a protein with normal acid precipitability and slightly greater than normal heat stability, maps at the left end of the A region but recombines with the heat labile mutants with a frequency less than 0.01 per cent.

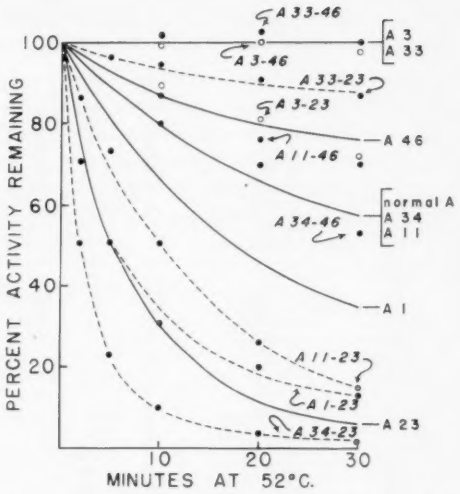

Fig. 5.—Heat inactivation of doubly altered A proteins. The A activity of initial extracts was measured in reaction (1) in the presence of excess normal component B. The inactivation curves for the various single mutant parent proteins are represented by the solid lines. For those double mutant proteins that do not resemble either parent protein or any other single mutant protein, the inactivation curve has been plotted using dashed lines. At least two extracts of each double mutant strain have been examined.

Those mutants that produce altered proteins with identical properties and do not appear to be genetically separable could represent repeat events at a mutational hot spot. Additional information relevant to this possibility has been obtained by testing the ability of a suppressor isolated in one A mutant strain to suppress the mutant phenotype of other A mutants. Suppressors of five of the heat labile strains cross-suppress all of the heat labile strains in the same cluster, but not the closely linked mutant *A 46* or any of several other A mutants that have been tested. In contrast, a suppressor of *A 3* does not suppress *A 33*, which maps at the same site as *A 3* and makes a heat resistant protein similar to the *A 3* protein, nor does this suppressor act on any other A mutant that has been tested. It can be concluded from these results that all of the clustered heat labile mutants are probably identical, but that the two mutations leading to heat resistant proteins, although they may have occurred at the same site, are probably not identical in kind and may have caused the substitution of different amino acids.

Another piece of evidence that the clustered heat labile mutants are all identical and may, in fact, involve the same amino acid change comes from the work of Helinski and Yanofsky,[19] who have shown that the proteins produced by a number of these heat labile mutants show the same peptide difference from the normal protein. On the other hand, the heat stable protein produced by mutant *A 33* has a readily detectable peptide difference that is not found in the heat stable protein produced by the genetically inseparable mutant, *A 3*.

Double mutants: Strains carrying more than one mutational alteration affecting

TABLE 4

RECOMBINATION TESTS FOR SEPARABILITY OF CLUSTERED SITES

A⁻ Donor (*hist*⁺ *cys*⁺)	A⁻ recipient (*hist*⁻ *cys*⁻)	*hist*⁻ *tryp*⁺ *cys*⁺ recombinants	Observed *cys*⁺ transductants	Corrected total*	Maximum recombination (*tryp*⁺ *cys*⁺/total) %
A 17	*A 23*	203	46,000	29,500	0.69†
A 23	*A 23*	0	37,000	23,700	...
A 24	*A 23*	0	337,000	216,000	<0.0005
A 27	*A 23*	0	122,000	78,200	<0.002
A 28	*A 23*	0	154,000	98,700	<0.001
A 35	*A 23*	0	234,000	150,000	<0.0007
A 36	*A 23*	0	238,000	153,000	<0.0007
A 46	*A 23*	0	40,000	25,600	<0.004
A 53	*A 23*	0	82,000	52,500	<0.002
A 17	*A 46*	27	58,000	34,100	0.08†
A 23	*A 46*	0	96,000	56,700	<0.002
A 28	*A 46*	0	279,000	165,000	<0.0006
A 35	*A 46*	1	544,000	321,000	0.0003
A 36	*A 46*	1	286,000	169,000	0.0006
A 46	*A 46*	0	81,000	47,800	...
A 53	*A 46*	1	73,000	43,100	0.002
A 3	*A 11*	0	76,000	50,200	<0.002
A 11‡	*A 11*	0	314,000	207,000	...
A 26	*A 11*	0	9,000	5,940	<0.02
A 33	*A 11*	0	39,000	26,700	<0.004
A 34	*A 11*	112	159,000	104,700	0.11
A 37	*A 11*	0	63,000	41,600	<0.003
A 41	*A 11*	0	313,000	207,000	<0.0005
A 48	*A 11*	0	50,000	33,000	<0.003
A 3	*A 3*	0	692,000	368,000	...
A 11	*A 3*	0	762,000	406,000	<0.0002
A 26	*A 3*	0	451,000	240,000	<0.0004
A 33	*A 3*	0	18,000	9,500	<0.01
A 34	*A 3*	162	228,000	121,500	0.13
A 41	*A 3*	0	145,000	77,200	<0.002
A 48	*A 3*	0	22,000	11,700	<0.009
A 1	*A 3*	0	253,000	139,000	<0.0007
A 34	*A 3*	157	288,000	153,000	0.10

* An estimate of the total number of *cys*⁺ cells that could have undergone recombination between the two *tryp*⁻ sites. Based on the number of *cys*⁺ transductants and the frequency of joint transduction of *cys* and *tryp*. This frequency was determined for each recipient and was 0.64, 0.59, 0.66, and 0.53 for the *hist*⁻ *cys*⁻ strains of *A 23*, *A 46*, *A 11*, and *A 3*, respectively.

† Although *A 23* and *A 46* are extremely close to one another, they often give widely discrepant values for recombination with *A 17*.

‡ The only strain producing an acid precipitable protein that was not tested by this method was *A 45*, but it had previously been found that *A 45–A 11* and *A 11–A 45* transductions give no *tryp*⁻ recombinants among approximately 100,000 transduced cells.

the A protein have been prepared by transduction. The preparation of these strains was greatly facilitated by the magnifying effect of transduction compared to conjugation on the recombinational length of a gene. Acting on the assumption that such double mutant strains would require tryptophan for growth, the following methods were employed for selecting and identifying these strains. Transductions of the type indicated were performed and plated on medium without

recipient:	Ax	+	cys⁻	hist⁻
donor:	+	Ay	cys⁺	hist⁺

cysteine but containing tryptophan and histidine. Only the cysteine-independent transductants will grow on this medium, and since *cys* and *tryp* are linked, this automatically selects cells that have been transduced by phage capable of transferring the A region. The double mutants resulting from recombination between the sites of Ax and Ay could comprise as much as 1 to 2 per cent of the cysteine-

independent transductions. These double mutants can be detected by their inability to yield tryptophan-independent progeny when transduced with phage grown on the single mutants Ax and Ay. A simplified spot test transduction procedure was used for the initial identification of possible double mutants. The putative doubles were then tested with each parent lysate employing the quantitative method of expressing tryptophan independence relative to transduction to histidine independence. The results of this test on all double mutants obtained to date are presented in Table 5. All of these strains, when tested with phage

TABLE 5

TRANSDUCTION TESTS OF DOUBLE MUTANTS

A⁻ Donor (*hist*⁺)	Double recipient (*hist*⁻)	No. *hist*⁺ transductants	*tryp*⁺ Recombinants— No. observed	No. expected*
A 1	*A 1-23* #1†	56,540	0	3,500
A 23	"	81,350	0	5,300
A 1	*A 1-23* #2†	39,600	0	2,450
A 23	"	25,520	0	1,650
A 3	*A 3-23*	3,360	0	208
A 23	"	940	0	45
A 11	*A 11-23* #1†	27,280	0	1,230
A 23	"	27,680	0	1,110
A 11	*A 11-23* #2†	27,120	0	1,220
A 23	"	31,040	0	1,240
A 13	*A 13-23*	3,300	0	145
A 23	"	780	0	32
A 3‡	*A 33-23* #1†	7,360	0	353
A 23	"	15,280	0	764
A 3‡	*A 33-23* #2†	13,800	0	662
A 23	"	15,680	0	785
A 34	*A 34-23*	12,530	0	480
A 23	"	9,800	0	360
A 3	*A 3-46*	1,020	0	51
A 46	"	1,280	0	63
A 11	*A 11-46*	720	0	15
A 46	"	200	0	10
A 14	*A 14-46*	16,280	0	180
A 46	"	1,960	0	24
A 33	*A 33-46*	6,120	0	275
A 46	"	5,020	0	285
A 34	*A 34-46*	10,200	0	387
A 46	"	36,200	0	1,170
A 14	*A 14-17*	9,900	0	230
A 17	"	7,260	0	140

* Calculated from parallel transductions using the corresponding single mutants as recipients.
† Different isolates.
‡ *A 3* is genetically inseparable from *A 33*.

grown on wild-type *E. coli K-12*, show a normal frequency of transduction to tryptophan independence relative to histidine independence and, therefore, the lack of recombination with either of the parent lysates indicates that both of the mutant lesions are present in these strains.

Properties of the double mutants: All double mutant strains were examined for the ability to produce a protein related to normal A protein and the properties of any such proteins were compared with the A proteins from the single mutant parents. These double mutants were initially tested for the presence of an altered A protein by measuring activity in reaction (1) (Table 6). It was found that those mutant sites that by themselves do not permit the production of any A protein capable of catalyzing reaction (1) also prevent production of detectable altered protein in a double mutant. Such doubles do, however, produce functional B

TABLE 6

ENZYMATIC AND IMMUNOLOGICAL ACTIVITY OF DOUBLE MUTANTS

A protein source	Specific activity* A	Specific activity* B	Ratio A/B	Relative neutralization by antibody to normal A
Wild type	2.9	2.7	1.1	1.00†
A 1-23 #1‡	0.93	7.2	0.13	0.52, 0.46
" #2‡	0.87	6.9	0.13	0.54
A 3-23	31	17	1.8	0.80, 0.93, 0.95
A 11-23	29	19	1.5	0.82, 0.80
A 33-23 #1‡	14	9	1.6	0.85
" #2‡	11	8	1.4	...
A 34-23	5.5	9.7	0.57	0.55, 0.55
A 3-46	17	12	1.4	0.80, 0.88
A 11-46	21	15	1.4	0.83
A 33-46	17	14	1.2	0.86
A 34-46	9.5	16	0.58	0.59
A 11-17	0.6**	38	...	...
A 13-23	1.0**	23	...	...
A 2-46	0.6**	32	...	...
A 14-46	0.5**	34	...	...
A 14-17	0.8**	24	...	...

* Units per mg protein in reaction (1).
† Normalized to 1.00 and all other values related to this.
‡ Different isolates.
** This activity can be attributed to the activity of B protein alone in reaction (1).

protein and are, therefore, still able to synthesize a protein involved in the same reaction. On the other hand, the double mutants produce an altered A protein that is active in reaction (1) in every case in which both of the parent strains also produce an enzymatically active A protein. The A/B ratio is normal (1.0–2.0) for most of these doubles, but three of the double mutants, *A 1-23*, *A 34-23*, and *A 34-46*, produce significantly less A than B activity. This reduction is most striking for the *A 1-23* double, which has an *A/B* ratio $^1/_5$ that of the *A 1* parent and $^1/_{10}$ that of the *A 23* parent.

It would appear that certain combinations of mutant sites either 1) decrease the production of A protein relative to B protein, 2) produce an A protein that is considerably more unstable during growth or isolation than the parent proteins, 3) produce a protein that combines poorly with B protein, or 4) produce a protein that is less effective with normal B per molecule than either normal A or the single mutant proteins. Some information that is relevant to these possibilities was obtained by examining the ability of the altered A proteins produced by double mutants to react with antibody to normal A protein. Only three double mutant proteins, those from *A 1-23*, *A 34-23*, and *A 34-46*, showed a pronounced reduction in the extent of neutralization by antibody. This suggests that either the interaction of these sites alters the configuration of that part of the molecule responsible for binding with antibody (and perhaps B) or each of these double mutant protein molecules forms an A-B complex that is less active enzymatically than complexes formed by normal or single mutant proteins. It is also possible that a fraction of the doubly altered protein loses enzymatic activity during growth or isolation without a corresponding loss of immunological activity.

Extracts of the two double mutants carrying the *A 11* site (*A 11-23* and *A 11-46*) were examined for precipitation of A activity at pH 4.0 (Table 7). The A activity of both of these doubles is precipitated by acid to the same extent as the A activity of *A 11* itself. On the other hand, extracts of double mutants that do

TABLE 7

ACID PRECIPITATION OF DOUBLE MUTANT PROTEINS

Mutational sites		Per cent activity* remaining after treatment of A protein from strains carrying the sites:		
x	y	A_{x-y}	A_x	A_y
11	*23* #1†	1.7	7	64
"	" #2†	4.1	"	"
11	*46*	4.3	3.8	71
3	*23*	71	70	69
3	*46*	65	70	71

* Activity in reaction (1).
† Different isolates.

not carry an altered site which alone results in the production of an acid precipitable protein (*A 3–23* and *A 3–46*, Table 7) lose no more A activity as a result of acid treatment than does normal A protein. Thus, it appears that acid precipitability in the *A 11* doubles is a result of the presence of the *A 11* mutation, which is freely expressed in proteins also bearing the *A 23* or *A 46* lesion.

The *A 23* site is also freely expressed in the *A 11–23* double mutant protein, for the A protein from this double is almost as sensitive to heat as *A 23* protein (Fig. 5). Thus, this double mutant protein has no properties that are not found in either of the parent single mutant strains, indicating that there is no obvious interaction of the two mutated sites in the protein. The heat sensitivities of the proteins from the single mutants *A 11* and *A 46* and the *A 11–46* double are all quite similar and, therefore, it is not possible to determine whether the double resembles one single mutant more than the other.

Double mutants carrying the altered sites from *A 3* and *A 33*, like the single mutants *A 3* and *A 33*, all produce altered A proteins that are more stable to heat than normal A protein, even when a mutation that confers heat lability to the A protein (i.e., *A 23*) is present in the same gene. It is possible that the mutations in *A 3* and *A 33* result in a change in the structure of the A protein such that the mutational alterations at the opposite end of the gene are not fully expressed.

One of the double mutant proteins showed an unexpected interaction of the two mutational changes. This protein, from the *A 34–23* double mutant, is extremely heat labile—much more so than the heat labile protein from *A 23*. Since *A 34* appears to be normal with regard to reaction to heating, the sensitivity of the doubly altered protein cannot be explained by the additivity of two alterations that both decrease the heat stability of the protein. Furthermore, if the effects of mutational change were generally additive, one would expect that the double mutant protein from *A 1–23* should be more labile than *A 23*, but this doubly altered protein is only as sensitive to heat as the *A 23* protein. It is clear from these results that in several cases the observed properties of the doubly altered proteins cannot be predicted from the properties of the singly altered parent proteins.

Discussion.—There is considerable evidence, presented here and elsewhere,[9, 15] that mutation within the A gene can lead to a variety of effects upon the protein controlled by this gene. The presence versus absence of an enzymatically or immunologically detectable A protein permits classification of A mutant strains into two major categories. Furthermore, within the group of mutants producing detectable A proteins many different types of protein alterations have been observed.

In the strains that produce altered A proteins the mutated sites are tightly

clustered at the two extreme ends of the A gene. In those strains that do not form such a protein the mutated sites appear to be distributed at random over the whole genetic region. However, several recently isolated strains that produce altered A proteins appear to have mutated sites located nearer to the center of the A gene. In spite of these few exceptions, mutations leading to altered proteins still occur predominantly at the ends of the A gene. The terminal localization of those mutational changes permitting the formation of altered A proteins may reflect the location of regions that constitute the active site of the enzyme. Intervening portions of the protein may be primarily concerned with maintaining the proper configuration of regions concerned with enzymatic activity.

The inability to detect recombination between mutated sites from different strains suggests that certain sites within the gene may be mutational hot spots. Moreover, the inseparability of the sites in strains *A 1*, *A 3*, *A 33*, and members of the *A 11* group indicates that several different changes may have occurred at the same site. These strains can all be distinguished either by the properties of the proteins they produce or by their reaction to suppressor genes. The conclusion that these alterations actually occurred at the same site depends entirely upon the sensitivity of the recombination test employed as a measure of genetic separability. This test allowed the examination of extremely large populations without the interfering effects of reverse mutation and in over half the tests where no recombinants were recovered values below 0.002 per cent could have been detected. The recombination frequency between the most widely separated known sites in the A region is approximately 2.5 per cent.[20] If this is a good approximation of the length of the A region, if all of the genetic information in this region is concerned with the coding of the A protein, and if the number of coding units required to specify different amino acids does not vary widely,[21] then the recombinational length of a segment of genetic material corresponding to one of the 280 amino acids in the A protein would be 0.009 per cent. Since recombination below 0.002 per cent could have been detected in many experiments, it can be concluded that, if the tested sites are actually genetically separable, more than four coding units would be required to specify each amino acid. In a few of the tests, where recombination frequencies below 0.0009 per cent could have been detected, more than 10 coding units would be required to specify each amino acid if the sites were separable.

These estimates are, of course, subject to the qualifications mentioned above and to the additional qualification that the recovery of prototrophic recombinants in the experiments using *hist⁻cys⁻tryp⁻* recipients appears to be lower than the recovery in experiments using *hist⁻tryp⁻* recipients. In spite of these objections, it can be tentatively concluded that two mutations inseparable by such tests probably represent changes at the same site. There is one complicating observation, however. Four different mutational changes appear to have occurred at the same site (in mutants *A 1*, *A 11*, *A 3*, and *A 33*). This observation is not compatible with the simple assumption that these mutations represent substitutions for the same nucleotide, since this assumption allows no more than three distinct mutant types resulting from substitutions at the same site if only the four common deoxynucleotides are involved in the genetic code of *E. coli*. However, mutant *A 33* does not revert and may, therefore, represent a change more extensive than a single nucleotide substitution. In general, these observations are consistent with

the assumption that several changes are possible at a single site and that the properties of an altered protein resulting from a change at a given site are, at least in some cases, dependent upon the nature of the change as well as its position.

All seven of the mutants mapping at the other prominent hot spot appear to be identical, for they all produce a heat labile A protein and can be suppressed by the same suppressor genes. Such a result would be expected if only one type of change occurs at this site, either because one of the several possible substitutions at this site is much more frequent than the others or because only one of these substitutions leads to a mutant phenotype. On the other hand, it is also possible that all substitutions at this site yield A proteins with the same properties. This would be reasonable if, for example, loss of stability were due to the removal of an amino acid essential for stability and none of the possible amino acid substitutions could restore this stability. This possibility appears unlikely, however, in view of the results obtained by comparing peptide patterns of digested A proteins from four of these apparently identical mutants. All four show the same peptide difference from the normal A protein,[19] suggesting that the A proteins from these four mutants carry the same amino acid substitution. It would appear from the inability to distinguish these mutants or the proteins produced by them, that the same mutational change may have recurred several times at the same genetic site.

A variety of interactions were observed in strains bearing two mutational alterations within the A gene, ranging from no effect on the physical properties characteristic of the parent proteins to interactions that give unique properties to the doubly altered protein. It will be interesting to examine the amino acid changes in these proteins to determine whether each of the primary structure changes of the parent proteins is present even in those cases where the properties of one of the parent proteins is masked. The ability to combine presumed amino acid substitutions in a protein should, in addition to aiding the demonstration of the autonomy of the effects of mutation on primary protein structure, contribute appreciably to our understanding of the intramolecular relationships that determine the characteristic properties of a protein.

Summary.—Altered A proteins formed by strains bearing different mutational changes in the gene governing the synthesis of the A protein of *E. coli* tryptophan synthetase can be distinguished from one another and from the normal A protein by their sensitivity to heat and acid. Two types of alterations recur frequently and appear to be due to identical changes at mutational hot spots. In addition, the available genetic evidence indicates that more than one type of change can occur at one of these hot spots.

Strains bearing two mutational alterations within the A gene have been prepared by transduction. The A proteins from some double mutant strains reflect the properties of the proteins formed by both parent single mutants, others resemble only one of the two parent proteins, and one double mutant protein exhibits unique properties that indicate some type of interaction between the two mutational alterations.

The implications of these results for further study of the relationship between gene structure and protein structure are discussed.

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[17] Because the number and type of recombinational events required to yield *tryp*$^+$ *cys*$^+$ progeny depends upon the order of the two altered sites in the tryptophan region, the recombination values presented in Table 4 can be used only as a qualitative measure of the ability of the two sites to recombine.

[18] For the purpose of this discussion the end of the A genetic region closest to the B genetic region has been arbitrarily designated the left end.

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[20] All recombination frequencies reported previously[15] relate the number of *tryp*$^+$ recombinants to the number of *hist*$^+$ transductants obtained from transductions using *hist*$^-$ *tryp*$^-$ recipients. Since the histidine region is transduced by phage Plkc with only $^1/_2$ the frequency of transduction of the tryptophan region, the true recombination frequency in the tryptophan region is only $^1/_2$ the ratio of *tryp*$^+$ recombinants to *hist*$^+$ transductants.

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PROGRESSIVE CONVERSION OF R-LOCUS EXPRESSION IN MAIZE

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Brink[1] reported unusual changes in maize *R*-locus expression in which (1) all *R* phenotypes were altered, (2) the change took place in one generation, and (3) the change was found to be genetic. The change (paramutation, induction, gene-conversion) takes place when *R* (responsible for pigmenting the aleurone layer of corn kernels) is made heterozygous with R^{st}. Brink[2] reported that the change in *R* was not progressive when *R* was left heterozygous with R^{st} for three generations.

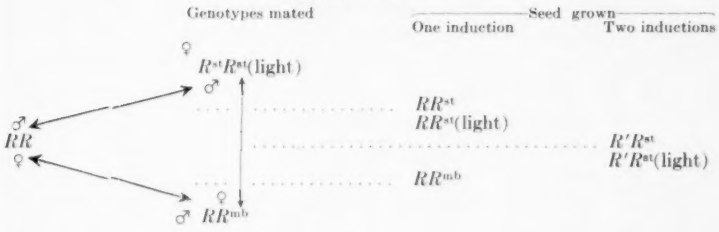
The present author considered that further tests for a progressive effect on *R* expression remained: the testing of different pattern alleles (R^{st}, R^{st} (light),

R^{mb}) on R successively. Tests performed under this last condition gave positive results and constitute the basis of this report.

Method.—To anticipate questions of modifier segregation, alleles backcrossed into the same inbred background were used in the following mating plan:

TABLE 1

MATING PLAN



Three plants in each of three families were intermated as indicated to the left of Table 1; both male and female inflorescences were used. R^{st} and R^{st} (light)—the latter phenotype is controlled by a modifier five units distal to the R^{st} locus—were incorporated into the same plant to insure a uniform background for comparison of the effects on R expression of both chromosome segments which condition the two stipple pattern expressions. Initial induction of R was supplied by an R^{mb} (marble pattern) known to be relatively weak in its effect on R. The RR^{mb} heterozygote was mated to the $R^{st}R^{st}$ (light) heterozygote for the second induction of R. The *same* plants of RR^{mb} and $R^{st}R^{st}$ (light) were also mated to the standard RR homozygote to demonstrate the magnitude of effect of each pattern allele, singly, on R and thus serve as experimental controls for the progressive effect when two different pattern alleles were used on R successively.

The demonstration of a progressive conversion of R-locus expression requires that the individual inducing alleles be relatively weak in their effect so that the pigment expressions fall centrally in the scoring ranges. The effect of any two alleles successively on R must then be demonstrably greater than that of any single pattern allele used.

R phenotypes were scored by matching samples of 50 kernels from each ear against a set of 22 standard kernels ranging from colorless to completely colored. R expressions before treatment—from RR homozygous, inbred stock—fell into classes 16–22 and thus lie outside the range of scores in this experiment. The relatively large number of standard kernels was used to provide increased resolution of smaller differences which have passed unnoticed with previous scoring procedures. Pigment values are reported as ear means based on 50 scored kernels. All R expressions were read on the same colorless, inbred W-23 background.

Results and Discussion.—Table 2 shows that the R alleles which were subjected to one and two induction treatments have no overlapping ear means. With R^{mb}, the initial pattern allele, R pigmentation in the aleurone layer was reduced to a mean value of 13.0. With R^{st} and R^{st} (light) alone, R phenotypes were reduced to scores of 6.4 and 7.9, respectively (scores based on ears from three separate families). However, when R^{st} and R^{st}(light) were made heterozygous with an R immediately extracted from an RR^{mb} heterozygote, R'' (prime symbol used to indi-

TABLE 2

COMPARISON OF *R* EXPRESSIONS IN MAIZE ALEURONE FROM ONE AND TWO GENERATION HETEROZYGOTES WITH INDUCING ALLELES

Parent	One induction scores ($\bar{X}$/50 kernels)	Parent	Two induction scores ($\bar{X}$/50 kernels)
	12.7		2.1
	4.9		1.1
RR^{st}	3.8	$R'R^{st}$	2.6
	4.5		1.3
	8.5		2.1
	3.8		2.5
			1.1
	$\bar{X}$ 6.4		$\bar{X}$ 1.8
	6.7		1.8
	8.3		1.3
	4.7		1.9
RR^{st}(light)	9.5	$R'R^{st}$(light)	2.4
	10.2		1.8
	7.8		1.6
	10.8		1.6
	8.2		1.9
	5.1		1.4
	8.8		1.5
	7.1		
	$\bar{X}$ 7.9		$\bar{X}$ 1.7
	13.9		
RR^{mb}	12.6		
	11.5		
	14.0		
	$\bar{X}$ 13.0		

cate the number of generations *R* has been heterozygous with a pattern allele) dropped to mean values of 1.8 and 1.7, respectively. The result, therefore, is an effect on the *R* phenotype, after the second treatment, greater than that produced by any of the pattern alleles individually.

That *R* undergoes progressive conversion from generation to generation is evidenced in several other ways. In three families in which *R* had been heterozygous with all three pattern alleles successively (each giving stronger initial induction than those used above), *75 per cent of the kernels after this third treatment of R were found to be colorless when R was tested through the pollen* (based on scores from eight ears, 50 kernels/ear).

After one generation of contact with a pattern allele, *R'* expressions regularly revert toward the level of pigmentation of the untreated *R*.[2] After two generations of contact, *R''* did not show this expected reversion. Instead, the *R''R''* homozygote gave ear means of 0.3 when tested through the pollen (nearly colorless phenotypes). Likewise, *R* alleles which had undergone progressive conversion were noted to give increased numbers of mottled kernels when brought through the female where two *R* chromosomes were contributed to the triploid endosperm tissue. Data for *R'''* (three contacts with pattern alleles) show that *R'''R'''* plants which had been selfed and thus had three *R'''* alleles present in the endosperm give a very high proportion of mottled kernels. Normally all kernels would be completely pigmented. Kernels, carrying three *R'''* alleles, begin to look like *R'* kernels where but a single *R'* allele is present. Data from five ears showed: 355 kernels completely colored while 575 of the kernels from those five ears ranged

from mottled to nearly colorless. Those same $R'''R'''$ homozygotes when tested on the recessive background for observation of R''' in one dose, were colorless or gave a few very weakly pigmented kernels.

The published record, Brink[1] and Brink and Weyers,[3] can be examined for support of the above data on progressive changes in R expression when R is maintained in contact with pattern alleles for several generations. It was noted[1] that phenotypes of R from the RR^{st} heterozygotes were not reverting in heterozygotes with r as expected. In both papers, it was pointed out that the $RR(R'R')$ homozygotes, derived from selfing RR^{st} heterozygotes, uniformly showed reversion toward normal pigment expression. It was reported in both papers that the heterozygote was selfed to produce the $R'R'$ homozygote and the $R'R^{st}$ heterozygote. The treatments of R were not the same. The $R'R'$ homozygote from this mating had been heterozygous with R^{st} or R^{mb} but one generation; the heterozygote ($R'R^{st}$ or $R'R^{mb}$) from the same selfed plant will yield R'' alleles. The data summarized in the present paper show that the R' and R'' *should* behave differently. Data in the above papers showed R'' gave less reversion in the presence of r.

It was noted in each of the above publications that $R'R'$ homozygotes gave completely pigmented kernels when selfed. The present paper calls attention to the reduction of pigment after progressive alteration of R for two or more generations. The end point of the process of pigment reduction is not known but presumably is the completely colorless, recessive, phenotype.

Because of the diversity of scoring procedures employed in the reporting of the results for the R-locus work, it is not possible to use the published scores for a rigorous support of the progressive changes in R expression. It is possible to note, ignoring the difference in inbred tester lines, that the color index scores reported[1] for R alleles coming from RR^{st} heterozygotes which had been selfed (and therefore were R'') yielded index scores very close to 3, whereas scores of 9.3 are reported[2] for the R' alleles from RR^{st} scored by the same technique as above.

A report on the induction effect on the plant color component of the R locus[4] is based on the measurement of the amount of pigment in R'''' and R''''' (alleles receiving three and four induction treatments) seedlings. The present author believes the successful demonstration of the induction effect on both anther and seedling pigment was due to the multiple treatments of the R-locus components, since a subsequent attempt to demonstrate the plant color effect on R' was unsuccessful.

Based on the present knowledge of the behavior of the R locus alleles, a satisfactory explanation for the contradictory results of the present paper and those of Brink,[2] in which no progressive effect was found, cannot be made. Some differences in the two experiments may be noted. The controls used and reported by Brink were from inbred lines in which opportunity for divergence of inducing action of the R^{st} was available for three generations. Brink's experiment assumes genotypic uniformity (because of inbred background) from generation to generation for the alleles concerned. This is an assumption which the R locus work challenges rather sharply. That the pattern alleles can undergo changes in inducing ability was reported by Weyers.[5]

The experiment reported here attempted to take the possibility of "divergence" or variation in inducing action of pattern alleles into consideration. The same

plant was used as a source of control and test alleles. That is, when the second treatment of R was made, alleles from the same plant ($R^{st}R^{st}$(light)) were used to produce the second treatment of R' (from the RR^{mb} heterozygote) and at the same time were also mated to standard, untreated R stocks (see Table 1) to serve as controls.

Another difference between the two experiments was the use in the present experiment of pattern alleles which caused R phenotypes in the midregions of pigmentation. It was possible to show that R alleles which had been treated twice produced demonstrably less pigment than those treated once because the scoring was done in a range of pigmentation where there was opportunity for considerable reduction in pigmentation after the first treatment. The controls and treatments reported by Brink[2] show all scores in the same low pigmenting region. Attention, however, must be directed to the index score of 3 reported[1] for R'' and subsequent reports of index scores of 9 for R'.[2]

It is difficult to evaluate R^{st} effects on R from the published results, since each report offers the scores by a different method. A standardized scoring system which utilizes the full range of variation present in the R' phenotypes is indispensable. In the R-locus work, this lack of a practical, standardized scoring system across the range of phenotypes has contributed greatly to the oversight of the progressive induction phenomenon.

That no special attributes need be assigned the different pattern alleles, i.e. pseudoallelism, became apparent when RR^{st} ears which had undergone selfing were scored. Scores alone could identify R pedigrees with R^{st}. R alleles which had been in contact with R^{st} for two or more generations scored one or more class intervals lower than those which had been heterozygous for just one generation.

The progressive effect on the R locus expression parallels that reported by Renner for *Oenothera* and summarized recently.[6] In support of Renner's findings, maize offers an inbred background with relatively well known and widely studied linkage groups. More recently, Sandler and Hiraizumi[7] reported progressive changes in connection with the *SD* locus of *Drosophila*. Brink's report[2] for no progressive changes in R expression must now be questioned.

The possibility for progressive changes in genetic units from generation to generation has far-reaching implications for genetic theory. But, as Stadler has pointed out,[8] classical genetics at this time is not prepared for this possibility.

Summary.—A progressive change for the R-locus expression was demonstrated for the R allele when it was left heterozygous with a pattern allele for two or more generations. After three generations of heterozygosity with pattern alleles, 75 per cent of the kernels carrying R were found to be completely colorless in testcrosses to recessive inbreds. Where R alleles are present in triploid aleuron tissue in three doses, three induction treatments made most of the kernels mottled or nearly colorless. R alleles which had undergone progressive induction were noted to give practically no reversion when homozygous. The finding of progressive changes in R can now explain previous anomalies reported for the R locus.

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AN XXY MALE IN THE MOUSE

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It has been known for two years that mice with an XO sex-chromosome constitution are fertile females;[1, 2] that human XO's are females, but usually infertile and afflicted with gonadal dysgenesis;[3] and that human XXY's are afflicted with the Klinefelter syndrome (small testicles, sterility, variable endocrine disorders). Klinefelter males occur relatively frequently in human populations.[4]

In the mouse, although the markers used in crosses for the detection of XO would, in most cases, also have led to detection of XXY (provided this type was male or a male-like intersex), no such animals were found in the original experiments.[2] Large numbers of animals obtained by one of us (LBR) in subsequent experiments, in which the crosses were marked for the detection of XXY, also did not yield this type. However, the conclusion that occurrence of the XXY condition was very much rarer than XO could not be definitely drawn, since it was possible that, for one of several reasons, XXY animals were not detectable. Thus, XXY might be female (as suggested by Hauschka *et al.*[5]) or a female-like intersex; or XXY might be inviable; or the expression of the sex-linked markers used might be altered by the presence of the Y.

McLaren[6] reported a mouse presumed to be XXY on the basis of its phenotype. This animal, which was of very stunted growth, died at 25 days of age, and no cytological proof of the XXY condition was obtained. Without this proof, and taking a conservative point of view, one could not absolutely rule out the possibility that the animal might, in fact, have been XY, since the particular phenotype on which its classification as XXY rested is sometimes mimicked by developmental disturbances associated with stunted growth. This left the situation rather unsatisfactory for the drawing of broad conclusions.

Doubts have now been dispelled by our finding, discussed in this paper, of an XXY mouse detected by sex-linked markers and studied cytologically. The evidence obtained from this animal, along with the thereby increased likelihood that McLaren's mouse was, indeed, XXY, and with Cattanach's[7] finding of presumed XXY (involving an X-rearrangement), shows that XXY in the mouse is a viable male and that Y does not affect the expression of sex-linked markers. With this knowledge now firmly established, a number of conclusions can be drawn from

our data concerning the role of the Y, the frequency of XXY, and the times of occurrence of nondisjunction.

Materials and Results.—The XXY animal arose in a cross of $+\ sf/+\ +\ ♀ \times Ta\ +/Y\ ♂$ (*sf* = scurfy; *Ta* = Tabby). Animals normally expected from such a cross are, phenotypically: striped females (*Ta*/+), wild type males, and scurfy males which die at 3–4 weeks of age. The exceptional animal is a male exhibiting a striping pattern exactly like that characteristic of *Ta*/+ females. His presumed genotype is $+\ sf/Ta+/Y$ or $+\ +/Ta+/Y$.

Although the exceptional male arose during the course of a radiation experiment, the circumstances show that the XXY condition must have been of spontaneous origin. The experiment involved postfertilization irradiation of his mother,[8] i.e., the XXY male was irradiated as a zygote. Since, however, two of his three sex chromosomes are of paternal origin (namely, X^{Ta} and Y), while his autosomes are diploid (see below), the abnormal event must have occurred in the father, rather than postfertilization.

The exceptional male is of normal size (weight at 3 months of age: 25.7 g; four female littermates: 19.8, 21.0, 21.1, 22.4 g, respectively; the one male littermate was *scurfy* and died at 3 weeks). His external genitalia are normal, testes can be palpated but may be small. He has been mated to nine females, namely, two (101 × C3H)F_1, one C3H, two *Blo*/+ and four *Blo*/*O* (*Blo* = Blotchy, a new sex-linked dominant, viable in the male[9]). Copulation has taken place repeatedly, as evidenced by vaginal plugs. However, to date, with the male $4^1/_2$ months of age, none of the females is pregnant and the XXY male may be presumed to be sterile. No sperm was found in the uterine contents of two females sacrificed shortly after they had copulated with the XXY male.

Chromosome analyses were made on colchicine-blocked, well-spread, Feulgen-stained metaphases in primary tissue cultures derived by tail biopsy taken from the exceptional male at the age of three weeks. Of 30 mitotic metaphase cells analyzed, 28 cells had 41 chromosomes (the remaining two had 82 and 38 respectively), including a Y chromosome. The autosomes were diploid and apparently normal. The karyotype of the exceptional male is illustrated in Figure 1. Other workers have not definitely identified the Y in the mouse. Stich and Hsu[10] have used as diagnosis of sex the presence of three of the smallest acrocentrics in the male as against two in the female. As, however, was shown by Chu and Monesi,[11] the Y is positively identifiable as the smallest element of the complement, acrocentric, and without a short arm. It is distinguishable from the members of the smallest autosome pair, which are slightly bigger and, though also acrocentric, are marked by the presence of small short arms. The identification of the X chromosome in somatic cells of the mouse is as yet uncertain. It may rank fifth or sixth in the descending order of length, in agreement with the observations of Ohno *et al.*[12] on spermatogonia.

Discussion.—The finding of a mouse which has a clear XXY karyotype and for which the genetic evidence shows that it must be of an X^MX^PY constitution (X^M and X^P being the maternally and paternally derived X's, respectively) has a number of implications.

It may be concluded that XXY in the mouse is viable and of normal size. Thus, if the case reported by McLaren was an uncomplicated XXY, its stunted growth

and early death may have been caused by other factors.

The fact that XXY is male confirms the male-determining properties of the Y in the mouse. The infertility of this male could indicate one of several things: either there are at least some female-determining factors on the X which, when added in double dose to the totality of female determiners, disturb the male-determination of the Y; or, there are dosage phenomena of genes not necessarily directly concerned with sex determination (i. e., the presence of products of two

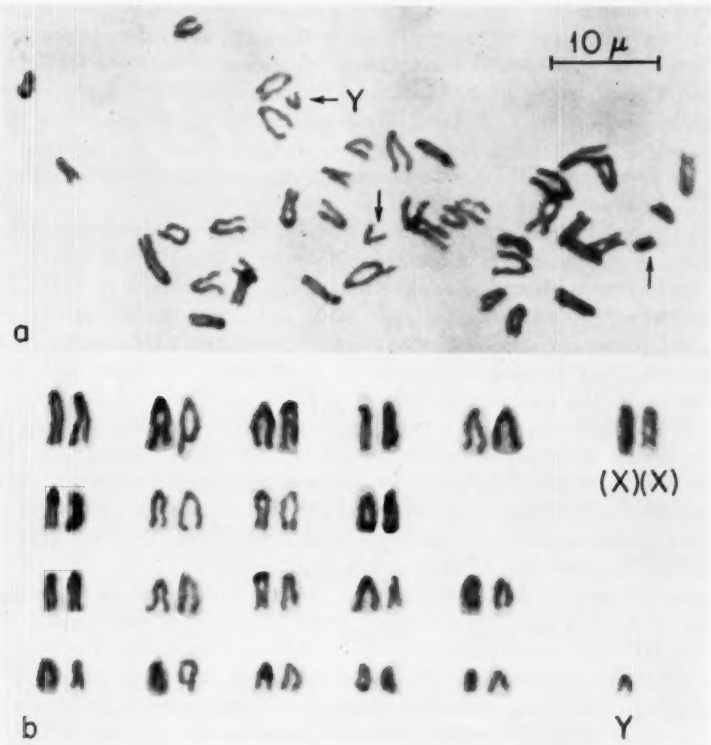

FIG. 1.—(*a*) A metaphase cell in tissue culture derived from tail biopsy showing 41 chromosomes. The Y chromosome is labeled. The unlabeled arrows indicate the smallest autosome pair. (*b*) The chromosomes of the same cell arranged in pairs showing the presumed X-chromosome pair and the Y chromosome.

sets of X-linked genes in a Y-determined male-developmental system); or, perhaps least likely, X and Y have a homologous region, and trisomy for this region leads to certain defects which result in infertility.

The fact that the phenotype of the $X^{+}X^{Ta}Y$ male is indistinguishable from that of $X^{+}X^{Ta}$ females indicates that the Y does not affect the expression of *Ta* in the heterozygous state. Originally, it was not inconceivable that $X^{+}X^{Ta}Y$ might be wild type if the Y had a suppressing effect, since the *Ta*/+ phenotype could be classified as a type of mottling, and since mottling due to another cause—namely, V-type position effect—occurred in females only.[13] However, it has recently been shown that *two* X's are required for the expression of the V-type position effect, and that

absence of mottling in normal males is due to presence of but a single X rather than presence of a Y.[14, 15] This conclusion is well in accord with the present finding of lack of effect of Y on the *Ta*/+ phenotype. If Tabby can be considered to be a dosage-compensated gene, then the phenotypic similarity of +/*Ta* and +/*Ta*/*Y* confirms Stern's conclusion[16] that "dosage compensation is founded on modifiers in the X chromosome which act independently of the sex-developmental system."

It should be noted that the X^MX^PY individual here described is, to our knowledge, the only mammal reported to date in which the stage of nondisjunction is known to be meiosis in a presumably normal male. (Cattanach[7] observed nondisjunction of X and Y centromeres in males bearing a rearrangement which presumably could enhance nondisjunction.) XO individuals can be due to nondisjunction in cleavage, as can X^MX^MY individuals. In man, cases of the Klinefelter syndrome in which the derivation of the X's was marked by the gene for color blindness were of the X^MX^MY type.[4]

Since various factors which could have made XXY undetectable—inviability, female sex-type, suppression of markers by Y—are now ruled out, certain conclusions can be drawn about the relative frequencies of occurrence of XO and XXY. Because the exceptional individual was X^MX^PY, i.e. presumably sired by an XY-sperm, the proper comparison is with the frequency of X^MO individuals. Among approximately 6,000 young from crosses in which these two types could be scored simultaneously, the estimated frequency of X^MO occurrence is 0.76 per cent, and of X^MX^PY occurrence, based on the single male here described, 0.02 per cent. (The estimated frequency of occurrence differs from the actual numbers observed because allowance has been made for the fact that, in some crosses, there is a probability of only one-half for detecting one or both types.) The data will be described in detail elsewhere. However, it is already clear from the grand totals that X^MX^PY is rarer than X^MO by a very large factor.

This result leads to the conclusion that events which would produce *equal* frequencies of X^MX^PY and X^MO—namely, those leading to nondisjunction of the X and Y at the first meiotic division in the male—are very much rarer than events that yield X^MO without X^MX^PY. Events yielding X^MO but not X^MX^PY can occur during meiosis (e.g., anaphase lagging; or nondisjunction in the equational division, which would also produce presumably nondetectable XX and YY gametes); or they can occur at the first cleavage. Cleavage as a relatively frequent source of XO's in the mouse was already suggested by nonrandomness of the original XO data, e.g., the finding of XO littermates.[1, 2] The frequency of XO can be increased by irradiation shortly after fertilization.[8] Ohno, Kaplan, and Kinosita[17] also believe nondisjunction to be more frequent at cleavage since of 1,460 second meiotic nuclei examined, each contained either one X or one Y.

Not only is X^MO much more frequent than X^MX^PY, but X^MO is also much more frequent than X^PO.[18] A hypothesis that would fit both of these results is that there is a relatively high probability of loss of the paternally contributed sex chromosome some time between fertilization and completion of the first cleavage.

Summary.—An XXY mouse was detected by sex-linked markers and studied cytologically. The genotype is + *sf*/*Ta* +/Y or + +/*Ta* +/Y. The expression of *Ta* is not affected by the Y. The exceptional animal is viable, of normal size,

and of male phenotype, though sterile. Since X^{Ta} and Y are both derived from the father, the XXY male provides proof of nondisjunction in meiosis of the male mouse. This event is apparently rare compared with events that lead to sex-chromosome loss only (in meiosis or cleavage), as indicated by the relatively much smaller frequency of $X^{M}X^{P}Y$ (0.02%) than $X^{M}O$ (0.76%) in crosses where these can be detected simultaneously.

We wish to thank Dr. E. F. Oakberg for examining the uterine contents of females mated to the XXY male.

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*EXTRA-TERRESTRIAL LIFE**

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The concept that life might exist on other planets is an exciting idea, which has wide popular appeal. The case for extra-terrestrial life has been presented at every intellectual level, including comic books. The other side has had little attention. There is a real danger that popular wishful thinking may lead to ill-considered projects involving vast sums of money and much skilled technical and scientific manpower.

In this paper I wish to review some of the cogent limitations on life as we know it and to examine some of the fundamental reasons why these limitations exist. Then I shall discuss some of the important features of the planetary and lunar environments which strongly contraindicate the presence of life on the moon, on Venus, or even on Mars.

Let us consider first the effect of temperature. Although many organisms have only a limited temperature range, some—such as the blue-green algae—can withstand greater extremes. Blue-green algae have been reported growing in hot springs at temperatures approaching 100°C. Other organisms multiply slowly at temperatures close to 0°C. At much lower temperatures vital activity must cease as the cellular fluid becomes frozen. At very low temperatures many organisms could presumably be preserved indefinitely although they would not metabolize or grow. Many organisms are destroyed in the pasteurization of milk. Standard bacteriological sterilization technique calls for sterilization at 15 pounds steam pressure for 15 minutes. This procedure occasionally fails to destroy some spores. However, a longer period of exposure is successful. When exposed to dry heat, temperatures of 170°C are required if sterilization is to be accomplished in 15 minutes. Four mechanisms involved in sterilization can be recognized. Destruction at 60°C is probably due to denaturation of some proteins, and failure of thermophiles to be destroyed at this temperature perhaps reflects utilization of a different type of protein (e.g., collagen does not coagulate at 60°C). Sterilization at 120°C in saturated steam indicates destruction of some of the many hydrolyzable chemicals of the organisms such as proteins and nucleic acids. There are indications that oxidation has a major destructive role when sterilization is carried out in dry air.

A fundamental limitation is that of the instability of organic substances. Almost all organic chemicals are thermodynamically unstable and degrade at rates which in the region of interest are exponentially increased with small elevations in temperature. For instance, alanine, one of the most stable amino acids, is half destroyed in about 10 minutes at 300°C, in 10 hours at 250°C, and in 30 days at 200°C.[1]

This well-known dependence of reaction kinetics on temperature is also noted in the destruction of bacteria and spores[2–4] with rates changing by a factor of 10 for each 7° to 10°C in moist heat or for each 15° to 35°C in dry heat. Long exposures to temperatures below the conventional sterilization regime may result in destruction of the organism. For instance, one in 100,000 spores of the thermophilic facultative anaerobe *Bacillus stearotheormophilus* can survive 42 seconds of exposure to superheated steam at 177°C and only the same fraction survive after 4,680 minutes of exposure at 121°C.[5] The data suggest that comparable destruction would occur in 100 days at 100°C with dry heat.

The whole question of thermal stability of organisms merits more study, particularly observations of time-death curves for extended periods at lower temperatures than have hitherto been employed. However, it seems extremely unlikely that any present terrestrial form of life can withstand long exposure to temperatures above 120°C even under the most favorable conditions. Nor does it seem likely that through adaptation a thermally resistant strain might be evolved which would be strikingly more resistant than those now known.

Water is a second important requirement of living organisms. Although some organisms and spores successfully withstand desiccation and resume vital processes on rehydration, growth and reproduction occur only in the hydrated state. One of the most striking examples is the water requirement of roots of plants. Research[6] has shown that most plants will grow if the relative humidity of air in the

soil is between 98.91 and 99.83 per cent but not at 98.90 per cent, although at this lower humidity most plants remain alive.

For another example, moist meat is an excellent culture medium. However, if the meat is dried and maintained at a relative humidity of less than 30 per cent, bacterial attack ceases. Consider the fate of a small amount of physiological saline solution, when it is exposed to air of low humidity. Water, of course, is lost. Ultimately the volume diminishes until the solution is supersaturated and NaCl begins to crystallize out. If the relative humidity is less than about 30 per cent, the process continues until only dry solids remain. Protoplasm behaves in a similar manner, having no special affinity for water. At relative humidities of about 30 per cent and below, terrestrial living matter eventually loses nearly all its water.

It is a well-known fact that all growing and dividing cells are "juicy" and consist mostly of water. Why is water essential to living things? Many answers could be given. Dehydrated protoplasm is hard and stiff. How could chromosomal division and the other essentials of reproduction, including reorganization of cells, occur in the dry state? How could transfer of metabolites occur? Many synthetic steps seem to involve assembly lines on which metabolites are handed along. However, some small molecules move freely within the cell. The cellular fluid, which is mainly water, is a highway for rapid transfer of many substances. Life without water would be a slow process, limited by solid state diffusion. Or it might be another kind of life involving gaseous diffusion or a different cellular fluid.

Water also enters into many of the chemical processes of the cell as a reactant and as such is completely essential to metabolism. Many biological reactions such as hydrolysis of proteins, esters, carbohydrates, and nucleic acids involve liquid water as a reactant. These reactions often are characterized by small negative free energy changes. Thus, in the hydrolysis of proteins values in the range of $-3{,}000$ cal/mol have been quoted. In utilizing protein as food or in reorganizing protein into new configurations the organism can take advantage of this free energy change, and if a catalyzing enzyme is present the reaction occurs quickly. If liquid water is not present, the fugacity of water is diminished and the free energy released in these hydrolysis reactions can be changed substantially. For instance, if the partial pressure of water were 1/1000 that of saturation, free energy of hydrolytic reactions would be changed about $+4$ kilocal/mol at 20°C. In such an environment peptides would not hydrolyze but rather the reverse reaction would occur spontaneously. The amino acids and proteins would tend to react to form impossibly large molecules and the living system could not function unless a means were devised to prevent untoward polymerization.

The great capacity of organisms to adapt is often cited. Superficially, the difference between a bacterium and an elephant is enormous. However, when examined in the light of comparative biochemistry the contrasts blur. In detail most of the important features are virtually identical, including the principal amino acids, enzyme systems, and the nucleic acids. The capacity of microorganisms to adapt is likewise often cited, but the actual alterations in enzyme systems or nucleic acids (as a result of adaptation) are relatively trivial. Observation of the capacities of living systems on this earth provides no basis for optimism that

through adaptation terrestrial organisms could circumvent the facts of thermal instability or those of chemical thermodynamics.

Let us turn then to a consideration of the lunar and planetary environments. Mercury can be quickly dismissed. It has no atmosphere and the face exposed to the sun reaches 340°C, while the dark side approaches absolute zero. The planets more distant than Mars are too cold. Only the moon, Venus, and Mars deserve serious consideration.

The lunar surface presents a singularly hostile environment, with temperatures ranging from intense cold at night to 135°C at the lunar equator. There is essentially no atmosphere, but rather a very high vacuum. Intense lethal solar radiation penetrates to the lunar surface. No liquid water is present. Watson, Murray, and Brown[7] have estimated that the vapor pressure of water on the moon is 1.4×10^{-12} mm of mercury. This corresponds to 3.5×10^{-13} of the saturation vapor pressure at 0°C. Under these conditions free energy of reactions involving hydrolysis would be changed by nearly +16 kilocal, which would constitute an enormous driving force toward polymerization of proteins. If an organism were to accompany a lunar rocket to the moon, its prognosis would be indeed dubious. On exposure to the sun, unless completely encased in protective vacuum-tight space, its water simply would boil away and its organic components would be gradually destroyed by radiation and heat.

The moon is, of course, always close to us and relatively easy to study. Venus and Mars are not so readily observed, nor so definitely known. One measure of the difficulty of obtaining trustworthy information concerning composition of the planetary atmospheres is the disagreement among authorities concerning fundamental facts. For instance, Menzel and Whipple[8] have suggested that the surface of Venus might be completely covered by an ocean. Kuiper[9] maintains that there is no ocean and no liquid water at the surface of the planet.

There are features of the planetary environment, however, which seem well established and which have important bearing on the questions of extra-terrestrial life.[10]

Venus has an atmosphere and is covered by a dense cloud layer. These features are especially evident at inferior conjunction when Venus appears as a crescent and the horns of the crescent are extended to a variable degree. In contrast to the moon and Mercury, which have no atmosphere or clouds, Venus has a high albedo—a relatively high fraction of incident light is not absorbed. What is the composition of the atmosphere? Only one component has been quantitatively determined—carbon dioxide. The measurement of this component, which was a triumph of astronomy, was performed by Adams and Dunham in 1932.[11] Later workers were able to make an improved estimate of the carbon dioxide content, which is now generally accepted as totaling 1,000-meter atmospheres.[12]

Attempts to detect water or oxygen in the atmosphere of other planets are hampered by the absorption of light by these components in our own atmosphere. However, it is possible to take advantage of a Doppler shift in wavelength, which Dunham[12] describes in the following:

"A qualitative examination of the infrared spectra of Venus failed to reveal any components of the oxygen lines in the B band or of the water-vapor lines near 8,000 Å which could be attributed to oxygen or water in the atmosphere of Venus.

The radial velocity of the planet averaged 14 km/sec during the period of observing, and this would correspond to a separation of 0.37 Å . . . in the spectrum.

"On the basis of the present evidence, it seems unlikely that there can be more than 5 per cent (probably less than 2 per cent) as much of either oxygen or water vapor in the atmosphere of Venus as in the atmosphere of the earth."

Recently, Strong[13] has investigated the atmosphere of Venus using a telescope in a balloon. He reported finding a trace of water—approximately 19 microns water equivalent above the cloud layer. The probable error of his measurement, however, is almost as large as the effect observed. Interpretation of these findings is, of course, difficult, for they essentially pertain to that portion of the atmosphere above the clouds. We do not know how much water is beneath.

Owing to the continual presence of clouds high in the atmosphere of Venus, it is not possible to determine the temperature of the surface by measurements of radiation of wavelength near the maximum on the black body emission curve. The best estimate comes from measures at cm wavelengths far to the long wavelength side of the black body maximum. The temperatures reported for the surface of Venus are in excess of 300°C,[14] which is, of course, far too hot to permit terrestrial life or complex organic substances to exist. This high temperature seems acceptable to most astronomers. They point to the effectiveness of carbon dioxide and cloud cover in conserving heat in a way often called a greenhouse effect. Solar radiation is absorbed at the surface, which re-emits radiation of longer wavelength, mainly in the infrared. The blanketing atmosphere is relatively opaque to this radiation. Hence heat is conserved. A similar greenhouse effect is active on earth where the carbon dioxide content is only 2 per cent that of Venus.

We turn then to Mars, which has always been regarded as the likeliest candidate for extra-terrestrial life. Mars has an atmosphere and, though on the average is colder than earth, has temperatures which at least part of the time are suitable for life. Temperatures at the equator rise to 35°C during the day. At night they fall far below freezing since there is practically no cloud cover and greenhouse effect.

The atmosphere is relatively thin and the surface is visible most of the time. The quantity of gas in the atmosphere was determined most accurately by Dollfus,[15] who measured the light reflected by the planet and determined what part was reflected from the surface and what part was due to Rayleigh scattering in the gas. The value which is generally accepted is a pressure of 85 millibars at ground level. What does this atmosphere consist of? Our knowledge is incomplete. Kuiper[16] has shown that part of the gas is carbon dioxide. Some of it is doubtless A^{40} arising out of decay of K^{40}. The remainder is generally assumed to be largely nitrogen, although there is no direct evidence for its presence.

There is disagreement concerning a haze in the atmosphere of Mars which leads to absorption that increases rapidly at wavelengths below 4500 Å. At least three explanations have been advanced. Kuiper[17] states that the haze is due to very small ice crystals. Öpik[18] attributes the absorption to carbon-containing particulate matter. Another explanation, advanced by Kiess *et al.*,[19] is that the absorption is due to nitrogen peroxide.

Using techniques similar to those which were successfully employed in the study of Venus, Adams and Dunham[20] searched for oxygen and water on Mars. Again

the technique of exploiting the Doppler shift was employed. No oxygen or water could be detected. An upper limit was set as 0.0015 as much water vapor above the surface of Mars as there is above Mt. Wilson on an average clear night in winter. Interpretation of the experimental results has been discussed by Vaucouleurs,[21] and he concluded that a reasonable upper limit for the amount of precipitable water vapor in the equatorial areas of Mars is 0.015 that above Mt. Wilson or 0.1 mm water.

Another approach to the question of water on Mars is to consider behavior of the polar caps which form in winter and disappear as summer approaches. Knowing the solar constant one can estimate the amount of heat falling on the cap and causing its evaporation. On the assumption that the cap is ice, Vaucouleurs[22] estimates that the cap averages 1 cm in thickness over an area of 10 million square kilometers. He thus obtains a value of 10^{17} grams for the total water content of Mars. This is only about one millionth the quantity present on earth. Assuming this amount of water to be uniformly distributed over the whole area of the planet, the maximum amount of precipitable water vapor is found to be 0.7 mm. He further states that it is quite obvious that at any moment the actual amount of water in the atmosphere must be only a small fraction of that condensed on polar areas, possibly less than one tenth. Thus he arrives once more at an atmospheric vapor content of 0.1 mm or less. Total atmospheric pressure at the ground is 85 millibars, or taking into account the lesser gravity of Mars the atmosphere has a mass 0.227 that of the earth. Thus on the average water vapor constitutes no more than one part in 20,000 of the atmosphere of Mars. On earth a typical comparable value might be of the order of one part in 100.

The Martian atmosphere then at best is an extremely dry one. Indeed, there is no certain evidence that free water is present on the planet, for water has never been detected there. The argument based on movement of the polar cap arbitrarily assumes that it is ice. Recently, for instance, Kiess *et al.*[19] have suggested that the polar cap might actually be nitrogen peroxide.

Mars is smaller than earth and correspondingly gases can escape far more readily. Only hydrogen and helium can leave earth, but it has been calculated that even atomic oxygen can escape from Mars.[10] It would be a most remarkable circumstance if we were to be especially favored with the privilege of being around to witness escape of the last traces of original water from Mars. It has been suggested that water could be supplied by planetary outgassing. At best such a supply would be sporadic. Volcanic activity on Mars has not been reported. In any event, the present moisture content of the Martian atmosphere is inadequate to permit life as we know it to grow and reproduce.

There is evidence, though not very convincing, of life on Mars. The well-known seasonal pattern of color variation has often been interpreted as of biologic origin. Following dust storms, earlier color reappears. Sinton[23] has measured the infrared spectra of light reflected from Mars and suggested that his spectra indicate the presence of organic molecules. The effects observed are small, however, and Kiess *et al.*[19] consider that they can also be explained as due to nitrogen oxides. If these oxides are present in substantial amounts at the surface of Mars they would constitute a toxic hazard to any terrestrial life which might reach Mars.

If life actually exists on Mars it cannot be like any terrestrial form of life because

of the relative absence of water. The crucial difficulty is the inability of life to function in a nonfluid state. If there is life, special arrangements to provide fluidity must be available, e.g. organic liquids of low vapor pressure. Such a form of life would be quite different from anything we know and would probably be destroyed in any standard culture medium.

In summary then it is quite unlikely that terrestrial-like life exists on the moon, Venus, or Mars. It is virtually impossible to contaminate these bodies by organisms carried from earth along with space devices.

One cannot be so certain that no life exists on Venus or Mars. If it is present, its detection will probably require methods other than those employed in microbiological laboratories. Successful meeting of the new challenges of space biology will demand a more imaginative, sophisticated approach than has been evident so far. Needed in the immediate future is a more solid base of certain and detailed information concerning the planetary environments. Of first priority are determination of a more certain value of the surface temperature of Venus and the composition of the atmosphere of Mars, especially water, carbon, and nitrogen compounds.

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*UNIFORM CONVERGENCE OF MONOTONE SEQUENCES OF FUNCTIONS**

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A Theorem of U. Dini states that on a bounded closed interval, or more generally on any compact space, if a monotone sequence of real-valued functions is convergent and the limit function is again continuous, then the sequence converges uniformly.

Recently, Luigi Amerio[1] has given a remarkable adaptation of this theorem to Bohr almost periodic functions on the open line, but Amerio's own version is such that the two theorems refer to functions on different spaces. Now, we will merge the two theorems into a single one, with hardly a change in proof required. The only shortcoming in our merger will be this, that Dini's theorem will be subsumed not for compactness in all generality but for sequential compactness only. In compensation, however, in Amerio's case, a somewhat weakened concept of almost periodicity will be sufficient. We will designate this weakened concept as "almost automorphy" because we have been encountering it first, and on several occasions then, in the (differential geometric) study of automorphic functions on real and complex manifolds.[2]

We will not enter into Amerio's application of his theorem, to which we have already contributed,[3] because matters there are still too much in flux.[4]

We take a triple of objects

$$[T; A; S]$$

of the following description. T is a general space without topology. S is a subspace. A is a group of one-to-one transformations of T, and for any y in T there is a point x in S and an element α of A such that $y = \alpha x$. Furthermore, S is endowed with a topology in which it is sequentially compact.

Dini's case arises if A consists only of the identity element and $S = T$, and Amerio's case arises in the opposite case if A is transitive on T and S consists of a single point.

We need consider only real-valued functions $f(x)$ on T. We call $f(x)$ *almost automorphic* if any infinite sequence of elements of A,

$$\alpha_1, \alpha_2, \alpha_3, \ldots \tag{1}$$

(repetitions allowed) contains an infinite subsequence

$$\beta_1, \beta_2, \beta_3, \ldots \tag{2}$$

having the following properties:

(i) The sequence of functions

$$f(\beta_1 x), f(\beta_2 x), f(\beta_3 x), \ldots \tag{3}$$

is convergent on T, and if we introduce its limit function

$$f_\beta(x) = \lim_{k \to \infty} f(\beta_k x),$$

then the sequence $\{f_\beta(\beta_k^{-1}x)\}$ is again convergent and its limit is the original function itself,

$$\lim_{k \to \infty} f_\beta(\beta_k^{-1}x) = f(x). \tag{4}$$

(ii) The family of functions (3) is jointly bounded on S,

$$|f(\beta_k x)| \leq G, \quad x \in S, \quad k = 1, 2, 3, \ldots$$

(iii) The sequence of functions (3) converges on S sequentially-continuously. That is, if

$$x_k \in S,\ x_0 \in S,\ x_k \to x_0 \tag{5}$$

then,

$$f(\beta_k x_k) \to f_\beta(x_0).$$

Remark 1: In defining almost periodicity proper (as against almost automorphy), the requirement would be that (3) shall converge uniformly on T. In this case, (4) is automatically fulfilled and the limit is again uniform.

Remark 2: The function $f(x)$ is continuous on S. In fact, since repetitions in (1) are allowed, we may put $\alpha_1 = \alpha_2 = \ldots = e$, where e is the identity element of A. In this case, (5) implies $f(x_k) \to f(x_0)$ and this is continuity.

Remark 3: $f(x)$ is bounded on T. Otherwise, there would be a sequence of points $\{y_m\}$ with $|f(y_m)| \to \infty$. On putting $y_m = \alpha_m x_m$, $\alpha_m \in A$, $x_m \in S$, and then introducing the function $f(\alpha_m x)$, we would obtain a contradiction to (ii).

Remark 4: If there is given a (finite or countable) sequence of almost automorphic functions $\{f_n(x)\}$, then by the diagonal argument any sequence $\{\alpha_m\}$ contains a subsequence $\{\beta_k\}$ for which the properties (i), (ii), and (iii) are fulfilled simultaneously. Especially, almost automorphic functions constitute a vector space over constants.

We now take a sequence of almost automorphic functions which is monotonely decreasing and bounded below,

$$f_1(x) \geq f_2(x) \geq \ldots \geq M > -\infty.$$

If $\{\alpha_m\}$, $\{\beta_k\}$ are as in Remark 4, we can introduce the limits

$$f_{n\beta}(x) = \lim_{k \to \infty} f_n(\beta_k x).$$

Obviously,

$$f_{1\beta}(x) \geq f_{2\beta}(x) \geq \ldots \geq M,$$

and thus we can introduce the limits

$$F_\beta(x) = \lim_{n \to \infty} f_{n\beta}(x). \tag{6}$$

In particular for $\alpha_m = e$, $\beta_k = e$, this introduces the limit function

$$F(x) = \lim_{n \to \infty} f_n(x).$$

We can now state our proposition:

THEOREM. *If for every such sequence* $\{\beta_k\}$ *the limit function* $F_\beta(x)$ *is again almost automorphic, then the original sequence* $\{f_n(x)\}$ *converges uniformly to* $F(x)$.

Remark 5: It follows easily from: $f_n(y) - f_{n+r}(y) \geq 0$ and

$$\sup_{x \in T}(f_n(\beta_k x) - f_{n+r}(\beta_k x)) = \sup_{x \in T}(f_n(x)) - f_{n+r}(x))$$

that then also every sequence $\{f_{n\beta}(x)\}$ converges uniformly to its limit $F_\beta(x)$.

Proof of the Theorem: By remark 4, we may replace in the proof of the theorem the sequence $\{f_n\}$ with the sequence $\{f_n - F\}$, that is, we may assume that for the original sequence we have

$$f_1(x) \geq f_2(x) \geq f_3(x) \geq \ldots \downarrow 0. \tag{7}$$

By remark 3, the numbers $\sigma_n = \sup f_n(x)$ are finite, and $\sigma_1 \geq \sigma_2 \geq \ldots \geq 0$. Therefore,

$$2\rho = \lim_{n \to \infty} \sigma \geq 0,$$

and our theorem will be proved if we show

$$\rho = 0.$$

There exists a sequence of points $y_n \in T$, such that

$$f_n(y_n) \geq \rho.$$

If we put $y_n = \alpha_n \xi_n$, $\xi_n \in S$, $\alpha_n \in A$, then there are indices $1 \leq n_1 < n_2 < n_3 < \ldots$ such that the sequence of elements $\beta_k = \alpha_{n_k}$ is as in Remark 4, and that furthermore the sequence of points $x_k = \xi_{n_k}$ is convergent in S, $x_k \to x_0$.

Since $n_k \geq k$ and $f_n(\alpha_{n+p} x_{n+p}) \geq f_{n+p}(\alpha_{n+p} x_{n+p})$, we have

$$f_n(\beta_k x_k) \geq \rho, \quad k \geq n, \quad n = 1, 2, 3, \ldots,$$

By property (iii), we therefore have

$$f_{n\beta}(x_0) \geq \rho,$$

and hence by (6), $$F_\beta(x_0) \geq \rho \geq 0. \tag{8}$$

Also, at all other points, (7) implies $f_{n\beta}(x) \geq 0$, and hence

$$F_\beta(x) \geq 0. \tag{9}$$

Now, since the theorem assumes that $F_\beta(x)$ is almost automorphic, the sequence $\{\beta_k^{-1}\}$ contains a subsequence $\{\gamma_r^{-1}\}$ for which there exists the limit

$$F_\beta(\gamma_r^{-1} x) \to G(x) \tag{10}$$

and then, in reverse, $$G(\gamma_r x) \to F_\beta(x). \tag{11}$$

From $f_{n\beta} \geq F_\beta$ and (10), we obtain

$$\lim f_{n\beta}(\gamma_r^{-1} x) \geq G(x), \; r \to \infty.$$

But by requirement (i), $$f_{n\beta}(\beta_k^{-1} x) \to f_n(x),$$

and hence, $$f_n(x) \geq G(x), \quad n = 1, 2, \ldots$$

By (7) therefore $0 \geq G$. However, (9) and (10) imply $G \geq 0$; therefore, $G = 0$. But then (11) implies $F_\beta(x) = 0$ for all x, especially $x = x_0$. Thus (8) gives $\rho = 0$ as claimed.

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SOME ASPECTS OF THE FERMAT PROBLEM (SECOND PAPER)

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In the first paper[1] under the present title, we considered methods of attack in attempts to prove that

$$x^\ell + y^\ell + z^\ell = 0 \tag{1}$$

is impossible where x, y, and z are nonzero rational integers and ℓ is an odd prime, and if x, y, and z are prime each to each.

As in our first paper, the conditions or criteria obtained failed so far to give any proofs of the Fermat theorem for special exponents. On the other hand, these relations give food for thought concerning the possibility or impossibility of (1). *In fact, in the present paper we reduce a number of known criteria for the solution of (1) involving cyclotomic integers to criteria involving rational integers alone. Hence we are able to compare the latter with some of the other previously-known results of the same type.*

1. We start our investigations with a statement of a theorem due to Kummer,[2] namely, that if $\mathfrak{p}(\zeta)$ represents an ideal in the field $k(\zeta)$, then $\Pi_s \mathfrak{p}(\zeta^{s_1})$ is a principal ideal, where $ss_1 \equiv 1 \pmod{\ell}$ and s ranges over the integers $h < \ell$ such that $h + |ch| < \ell$, where c is any integer with $(c(c+1), \ell) = 1$ and $|ch|$ is the least residue of ch modulo ℓ. We shall now apply this result in connection with (1). If $z \not\equiv 0 \pmod{\ell}$, then (1) shows that, as is known, with $i = l - 1$,

$$\left.\begin{aligned} (x + y) &= \mathfrak{a}_0^\ell, \\ (x + \zeta y) &= \mathfrak{a}_1^\ell, \\ &\cdots\cdots\cdots\cdots \\ (x + \zeta^i y) &= \mathfrak{a}_i^\ell, \end{aligned}\right\} \tag{2}$$

where the $\mathfrak{a}$'s are ideals in the ring of integers defined by ζ where

$$\zeta = e^{2i\pi/\ell},$$

since the principal ideals on the left are prime each to each. (In what follows, the ring just mentioned will be designated by R_ζ. Also, R_1 will denote the ring of rational integers. If a fraction such as a/b appears in an exponent of ζ, then it is defined as an integral solution k of $bk \equiv a \pmod{\ell}$.) Applying the Kummer theorem just mentioned to (2), we obtain, if $\omega(\zeta) \epsilon R_\zeta$,

$$\prod_s (x + \zeta^{s_1} y) = \zeta^g (\omega(\zeta))^\ell. \tag{3}$$

We shall now consider the value of g in (3). This can be obtained from the relation proved elsewhere,[3]

$$cq(c) - (c+1)q(c+1) \equiv \sum \frac{1}{s} \pmod{\ell}, \tag{4}$$

where s ranges as above. Here $q(c) = (c^{\ell-1} - 1)/\ell$. Set $\lambda = \zeta - 1$, and reduce the relation (3) modulo λ. This gives

$$(x + y)^{(\ell-1)/2} \equiv u^\ell \pmod{\lambda},$$

and therefore,

$$(x + y)^{(\ell-1)/2} \equiv u^\ell \pmod{\ell}, \tag{5}$$

where $(\omega(\zeta))^\ell \equiv u^\ell \pmod{\ell}$, $u \epsilon R_1$. We now reduce (3) modulo λ^2. The left-hand member gives

$$\begin{aligned} \prod_s (x + (\lambda+1)^{s_1} y) &\equiv \prod_s (x + y + s_1 \lambda y) \pmod{\lambda^2}, \\ &\equiv (x+y)^{(\ell-1)/2} + \sum_s s_1 y \lambda (x+y)^{(\ell-3)/2} \pmod{\lambda^2}. \end{aligned}$$

Reducing the right-hand member modulo λ^2 gives $(1 + g\lambda)u^\ell \pmod{\lambda^2}$, and employing (5) gives, noting that $ss_1 \equiv 1 \pmod{\ell}$,

$$\frac{y}{x+y} \sum_s \frac{1}{s} \equiv g \pmod{\ell}. \tag{6}$$

From equations (4) and (6), we find if c is an integer with $(c(c+1), \ell) = 1$,

$$g \equiv \frac{y}{x+y} (+cq(c) - (c+1)q(c+1)) \pmod{\ell}. \tag{7}$$

2. We shall now introduce a simple lemma which will enable us to derive criteria for the existence of (3) as well as somewhat similar relations involving x and y which we shall mention later.

Let, if each a and $b \epsilon R_1$,

$$\begin{aligned} a_0 + a_1\zeta + \ldots + a_{\ell-2}\zeta^{\ell-2} &= (b_0 + b_1\zeta + \ldots + b_{\ell-2}\zeta^{\ell-2})^\ell, \\ &= A_0 + A_1\zeta + \ldots + A_{\ell-1}\zeta^{\ell-1}, \\ &= A_0 + A_1\zeta + \ldots + A_{\ell-1}(-1 - \zeta - \ldots - \zeta^{\ell-2}), \\ &= (A_0 - A_{\ell-1}) + (A_1 - A_{\ell-1})\zeta + \ldots + \\ &\qquad (A_{\ell-2} - A_{\ell-1})\zeta^{\ell-2}. \end{aligned} \tag{8}$$

Whence

$$a_k = A_k - A_{\ell-1}, \tag{9}$$

$k = 0, 1, 2, \ldots, \ell - 2$, since, as is known, any element of R_ζ may be written uniquely in the form given in the left-hand member of (8).

We then have the

LEMMA. *If (8) holds, then (9) holds where the A's are obtained from*

$$(b_0 + b_1\zeta + \ldots + b_{\ell-2}\zeta^{\ell-2})^\ell,$$

on expanding this by the multinomial theorem and collecting the coefficients of each distinct power of ζ.

Take the relation (3) and assume that $y \equiv 0 \pmod{\ell}$. Then it becomes, after we use (6),

$$\prod_s (x + y\zeta^{s_1}) = \rho^\ell, \tag{10}$$

where $\rho \in R_\zeta$. Then the left-hand member may be put in the form of the left-hand member of (8) with each a being a homogeneous polynomial in x and y of degree $(\ell - 1)/2$. We may then apply our lemma and find relations of the type (9) with each a a function of the type just mentioned and the A's defined as in the lemma. Hence, we have a set of conditional equations in $\ell + 1$ quantities, x, y, b_0, b_1, $\ldots$, $b_{\ell-2}$ of degree ℓ. It is obvious that the steps in this argument can be reversed; hence, the conditions are necessary and sufficient for the solution of (10). Further, we shall now show that if we add the condition that $x + y = v^\ell$, the conditions just mentioned are also necessary and sufficient for the solution of (1). To do this, we note in (3) that s was defined as any integer prime to ℓ in the set $1, 2, \ldots, \ell - 1$, which satisfies the conditions $s + |cs| < \ell$. Further, $|cs|$ is the least residue of cs modulo ℓ. It is easy to show that if $s = k$ satisfies these conditions, then $\ell - k$ does not. Consequently, if we replace ζ by ζ^{-1} in (10), the resulting equation together with (10) gives $(x^\ell + y^\ell)/(x + y) = w^\ell$ with $w \in R_1$. And if we multiply this equation through by $x + y = v^\ell$, we obtain (1), if $vw = -z$.

We know[4] that if (1) is satisfied with $y \equiv 0 \pmod{\ell}$, then $y \equiv 0 \pmod{\ell^3}$. If we use this condition in the relations just obtained, they become much simpler when the whole relation is taken modulo ℓ^3; that is, the left-hand member of the conditional equations reduces in each case to the quantity $x^{(\ell-1)/2}$. So we then have a set of $\ell - 1$ conditional congruences each of degree ℓ, and involving ℓ unknowns with the modulus ℓ^3.

We now consider (3) with xyz prime to ℓ. Then since $y \not\equiv 0 \pmod{\ell}$, g is not necessarily $\equiv 0 \pmod{\ell}$ unless $-cq(c) + (c + 1)q(c + 1) \equiv 0 \pmod{\ell}$. If $(xyz,\ell) = 1$ in equation (1), we have[5] $q(c) \equiv 0 \pmod{\ell}$ for the primes $c < 44$ and therefore for the composite c's $\leqq 44$. For these particular values of c, we can then apply a method, using our lemma, to obtain a set of ℓ equations in $\ell + 1$ quantities, each equation of degree ℓ. These results can be summarized in the statement of the theorem:

THEOREM 1. *If (1) is satisfied with* $xyz \not\equiv 0 \pmod{\ell}$, *then for every* $c < 44$ *in (3) there exists a set of* ℓ *conditional equations in* $\ell + 1$ *unknowns, each of degree* ℓ, *which equations must be satisfied and which involve rational integers only. In particular, if* $y \equiv 0 \pmod{\ell}$, *then there exists*[6] *such a set for each c in (3) in the range 1, 2, …,* $\ell - 1$. *In the latter case, for a given c the conditions mentioned are necessary and sufficient for the existence of a solution of (1).*

From here on, we shall refer to the case when $(xyz, \ell) = 1$ in (1) as Case I of the

theorem and the case when $(xyz, \ell) \neq 1$ as Case II.

3. We shall now make comparisons with the results (see ref. 2, p. 563) in Theorem 1 with the Kummer criteria, which are as follows: If (1) is satisfied in Case I, then

$$B_n\left[\frac{d^{\ell-2n}(x+e^v y)}{dv^{2n}}\right]_{v=0} \equiv 0 \pmod{\ell}, \tag{12}$$

for $n = 1, 2, \ldots, (\ell-1)/2$ where B_n is the nth Bernoulli number, $B_1 = 1/6$, $B_2 = 1/30$, etc. Also, x and y in (12) can be replaced by any one of the pairs (x, z), (y, z), (z, y), (y, x), (z, x). By means of the Fermat quotient criteria,[5] D. H. and Emma Lehmer[7] in 1941 showed that (1) was impossible in Case I for all $\ell < 253,747,899$. *However, in effect, Kummer obtained his criteria (12) by considering (3) with respect to the modulus ℓ, whereas criteria in our Theorem 1 for Case I are all equations and will obviously give corresponding congruences with respect to any modulus whatever.* Also, the criteria in Theorem 1 are necessary and sufficient in Case II as was stated. In addition, from the Kummer criteria, relations have been derived which indicate that we have not yet exhausted its possibilities. As a particular example of this, let us take the congruences which were obtained from the Kummer criteria, stated elsewhere,[8]

$$\frac{t(t^m-1)}{t-1}\sum_{\rho}\frac{F_\ell(\rho)(1-\rho)}{m\ell(t-\rho)} \equiv 0 \pmod{\ell}, \tag{13}$$

where t has any one of the six values mentioned in connection with equation (12) of the present paper, where ρ is an mth root of unity $\neq 1$, and

$$F_n(j) = \sum_{r=0}^{\ell m-1} r^{n-1} j^r, \quad (n > 1). \tag{14}$$

Setting $m = 2$ and $m = 3$ in (13), we derive easily from this the criteria $q(2) \equiv q(3) \equiv 0 \pmod{\ell}$, but from (13) *we have left $\ell - 3$ congruences which must also be satisfied.* As far as I know, there is no value less than 26,000 which satisfies the first two mentioned. In view of the result of D. H. and Emma Lehmer already referred to, we may take ℓ in Theorem 1 as far as it refers to Case I and in the Kummer criteria as $\geqq 253,747,899$. *In spite of this and other results for special exponents which have been obtained by the use of the Kummer criteria in Case I, it seems quite possible, in view of our Theorem 1 and what we have just said about the possibilities of the Kummer criteria, that so far we have only been scratching on the surface of the problem.*

4. As to Case II of the Fermat theorem, we note that Theorem I gives more extensive criteria than it does in Case I. The particular merit of the use of relation (3) is that it is true for all cyclotomic fields defined by an odd prime ℓ. In particular, we do not have to make any provisions concerning the class number[9] of R_ζ. However, we shall now introduce the *second factor* in the class number of R_ζ and prove that we can obtain criteria somewhat similar to those given in Theorem 1 but much simpler, when this second factor is prime to ℓ.

The writer proved the following relation:[10]

$$\left(\frac{x+\zeta^k z}{\zeta^k-1}\right)\left(\frac{x+\zeta^{-k}z}{\zeta^{-k}-1}\right)^{\ell-1} = \gamma^\ell, \tag{15}$$

where $\gamma \epsilon R_\zeta$. In the same way that (15) was proved, we may show that, if $\sigma \epsilon R_\zeta$,

$$(x + y\zeta^{-1})(x + y\zeta)^{\ell-1} = \sigma^\ell, \tag{16}$$

when as before, $y \equiv 0 \pmod{\ell}$, since each factor on the left is primary in R_ζ. Proceeding with (15) and (16) as we did to prove Theorem 1, we shall obtain, from each of these two relations, a set of ℓ conditional equations in ℓ unknowns, each of degree ℓ, which must be satisfied, if (1) is to be satisfied in Case II, provided the second factor of the class number of R_ζ is prime to ℓ.

5. We shall now show that criteria concerning the divisibility of the second factor of the class number of R_ζ can be obtained which are somewhat similar to those given in Theorem 1 and those described in the last paragraph above. Also, these criteria involve rational integers only. Let $k_i = \zeta^{r^i/2} - \zeta^{-r^i/2}$ and $e_i = k_{i+1}/k_i$ with r a primitive root of ℓ. Set

$$E_n(\zeta) = E_n = \prod_{i=0}^{(\ell-3)/2} e_i^{r^{-2in}} \tag{17}$$

for n any integer in the set $1, 2, \ldots, (\ell - 3)/2$.

The writer proved[11] that the second factor of the class number of the ring R_i is divisible by ℓ if and only if at least one of the quantities E_i, $i = 1, 2, \ldots, (\ell - 3)/2$ is the ℓth power of a unit in R_ζ.

D. H. Lehmer, Emma Lehmer, and the writer showed that[12]

$$\zeta^{-d/2} \prod_{b=1}^{\mu} (\zeta^b - 1)^{b^{\ell-1-2n}} = E_n(\zeta)^m \delta^\ell, \tag{18}$$

where m is prime to ℓ, $\zeta \epsilon R_\zeta$, $\mu = (\ell - 1)/2$, and where

$$d = 1^{\ell-2n} + 2^{\ell-2n} + \ldots + \mu^{\ell-2n}.$$

Consequently, $E_n(\zeta)$ is the ℓth power of an element in $R(\zeta)$ if and only if the left-hand member of (18), say $F(\zeta)$, is the ℓth power of an element in R_ζ.

Let
$$F_n(\zeta) = \beta^\ell \tag{19}$$

with β a unit in R_ζ. If we reduce the left-hand member of this relation to the form

$$h_0 + h_1\zeta + \ldots + h_{\ell-2}\zeta^{\ell-2},$$

with the h's in R_1 and set, for each k in R_1,

$$\beta = k_0 + k_1\zeta + \ldots + k_{\ell-2}\zeta^{\ell-2},$$

then we may apply our lemma and obtain $(\ell - 1)$ conditional equations in k_0, $k_1, \ldots k_{\ell-2}$ of degree ℓ. Hence, the above-mentioned criteria for the divisibility of the class number of R_ζ by ℓ, which involves algebraic numbers, may be replaced by a set of $(\ell - 1)$ conditional equations in $\ell - 1$ unknowns, involving rational integers only. These conditions are necessary and sufficient for the divisibility of the second factor of the class number by ℓ, as the steps used in obtaining them are obviously reversible.

7. We shall now consider the results obtained from the use of a quite different method of attack on the Fermat problem from any method discussed in this or

our former paper. The results will be given without proof. Let a and b be any nonzero rational integers so that $(a, b) = 1$, $(ab, z) = 1$, and $(a^\ell + b^\ell, x) = 1$. Also, set

$$M = \frac{u^\ell + v^\ell}{u + v},$$

where $u = a^\ell x$, $v = (a^\ell y + b^\ell(x + y))$. Then if (1) is satisfied in nonzero integers with $(x, y, z) = 1$ and $y \equiv 0 \pmod{\ell^2}$, then M either has all of its divisors in the form $1 + k\ell^2$, or if a divisor exists in the form $1 + h\ell = q$, with $h \not\equiv 0 \pmod{\ell}$ and q prime, then M has the form $cq^{\ell a}$, with $c \not\equiv 0 \pmod{q}$. It is known that M has all its divisors in the form $1 + g\ell$ provided $u + v$ is prime to ℓ. Also, it can be easily shown that M has the form $1 + d\ell^2$. It is clear from the definitions of a and b that M ranges over an infinity of values. This result is a simple special case of some general criteria which have been obtained by the writer for the solution of (1) in the so-called Case II of the theorem.

* The author's work on this paper was done under grant 8238 awarded him by the Nationa Science Foundation.

[1] Vandiver, H. S., "Some Aspects of the Fermat Problem (First Paper)," these PROCEEDINGS, **47,** 202 (1961). In footnote 3 therein, insert the word "known" after "three."

[2] Vandiver, H. S., "Fermat's Last Theorem," *Amer. Math. Monthly,* **53,** 562 (1946). Cf. also the references to Weber and Landau there given.

[3] Vandiver, H. S., "On Symmetric Functions Formed by Systems of Elements of a Finite Algebra and Their Connection with Fermat's Quotient and Bernoulli's Numbers," *Annals of Math.*, **18,** 113–114 (1917), first relation below (12).

[4] Landau, E., *Vorlesungen über Zahlentheorie* (Leipzig: Verlag S. Hirzel, 1927), Bd. 3, pp. 327–328.

[5] These results were obtained by a series of writers. Cf. reference 2, this paper, p. 562.

[6] Repetitions exist in general for the set of s's in (10) for different values of c. Induction indicates that $(l - 1)/2$ of them are distinct for each c, but this question the writer has not cleared up as yet.

[7] *Bull. Amer. Math. Soc.*, **47,** 139–142.

[8] Vandiver, H. S., "Fermat's Last Theorem," *op. cit.*, 575–576.

[9] *Ibid.*, 567, 568. Note the following errata in that paper: On p. 568, relation (17a), replace the addition sign by a multiplication sign, and similarly for the relation at the top of p. 572. Also, on p. 571, add 389 and 613 to the list of irregular primes near the middle of the page.

[10] Vandiver, H. S., "On Fermat's Last Theorem," *Trans. Amer. Math. Soc.*, **31** (1929), relation (7a). In this relation, we may set $\omega = x$ and $\theta = z$, as ω and θ are integers in the field defined by ζ, which field includes R_1.

[11] Vandiver, H. S., "On the Second Factor of the Class Number of a Cyclotomic Field," these PROCEEDINGS, **16,** 743–749 (1930). A somewhat simpler proof was given by K. Inkeri, "Über die Klassenzahl des Kreiskörpers der ℓten Einheitswurzeln," *Annals of the Acad. of Sci. of Finland,* Series A, 5–7 (1955).

[12] Lehmer, D. H. and Emma, and H. S. Vandiver, "An Application of High-speed Computing to Fermat's Last Theorem," these PROCEEDINGS, **40,** 30 (1954), relation (17).

GENERALIZED INVARIANT IMBEDDING RELATION

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1. *Introduction.*—The invariant imbedding relation is a mathematical tool which has been developed to facilitate the formulation of neutron and photon transport problems on a large variety of geometric spaces.[1] The relation itself is a precise analytic version of the verbally stated principle of invariant imbedding formulated by Bellman and Kalaba.[2] The invariant imbedding principle was, in turn, motivated by the classical principles of invariance as developed by Chandrasekhar[3] from the initial idea of Ambarzumian.[4] The purpose of this note is to generalize the analytic version of the invariant imbedding relation to its most symmetric form for one-parameter spaces (e.g., spherical, cylindrical, plane-parallel spaces). The present generalized version of the relation is motivated by more than just symmetry reasons: It will be shown (*a*) that the $\mathfrak{M}$-operation of the new form possesses the analytically important group property and (*b*) that the generalization contains the basis for at least one new means of solving transfer problems.

2. *Statement of the Generalized Relation.*—Let X be a one-parameter carrier space in E_3; that is, X is a subset of E_3 of the form $X = \{X_y\colon\ a \leqslant y \leqslant b\}$ where X_y is the two-dimensional surface belonging to the parameter y, which ranges between fixed limits $a \leqslant b$. For example, $y - a$ may be the depth of X_y below the upper boundary X_a of a plane-parallel medium. In general, however, X_y is curvilinear. On each subset X_y of X, there is defined a radiance or specific intensity function $N(y)$ which, when a point p_y on X_y and a direction ξ are given, assigns a radiance $N(y, p_y, \xi)$ to the point p_y in the direction ξ. At each point of each level X_y in X, the unit sphere Ξ in E_3 is partitioned into an *upward* Ξ_+ and a *downward* Ξ_- hemisphere. To denote that the partition of Ξ depends on X_y, we write $\Xi_+(y)$, $\Xi_-(y)$. For example, in a plane-parallel medium, $\Xi_\pm(y)$ are independent of position in X_y and in fact, independent of y. In general, this partition of Ξ on each X_y induces a natural decomposition of the radiance function $N(y)$: The restriction of the function $N(y)$ to Ξ_+, denoted by $N_+(y)$, is called the *upward* radiance, and the restriction of $N(y)$ to Ξ_- is called the *downward* radiance. Let $\mathfrak{N}$ denote the family of all ordered radiance pairs $[N_+(y), N_-(z)]$, where (y, z) is an ordered pair of parameters; i.e., $(y, z) \epsilon [a, b] \times [a, b]$. We are now ready to state the

Generalized invariant imbedding relation: Let $X = \{X_y\colon\ a \leqslant y \leqslant b\}$ *be a one-parameter carrier space. To each pair of points* (u, v), (w, x) *in* $[a, b] \times [a, b]$ *let there correspond a pair* $[N_+(u), N_-(v)]$, $[N_+(w), N_-(x)]$ *of points in* $\mathfrak{N}$. *Then there is a map* $\mathfrak{M}(u, v; w, x)$ *of* $\mathfrak{N}$ *into* $\mathfrak{N}$ *such that*

$$[N_+(w), N_-(x)] = [N_+(u), N_-(v)]\, \mathfrak{M}(v, x; u, w) \tag{i}$$

$$\mathfrak{M}(v, x; u, w) = \begin{pmatrix} \mathfrak{I}(u, w; v, x) & \mathfrak{R}(u, x; v, w) \\ \mathfrak{R}(v, w; u, x) & \mathfrak{I}(v, x; u, w) \end{pmatrix}, \tag{ii}$$

and where the four $\mathfrak{I}$ and $\mathfrak{R}$ operations are the *extended transmittance and reflectance operators* respectively.

3. *Special Cases.*—The generalized invariant imbedding relation is a proper generalization of the standard invariant imbedding relation given earlier.[1] To see this, assume (*a*) that $w = x$, and (*b*) that $v \leqslant x \leqslant u$. Then it can be shown by specific, concrete examples, that

$$\begin{aligned}
\mathfrak{I}(u, x; v, x) &= \mathfrak{I}(u, x, v) \\
\mathfrak{I}(v, x; u, x) &= \mathfrak{I}(v, x, u) \\
\mathfrak{R}(u, x; v, x) &= \mathfrak{R}(u, x, v) \\
\mathfrak{R}(v, x; u, x) &= \mathfrak{R}(v, x, u)
\end{aligned}$$

and hence, that

$$\mathfrak{M}(v, x; u, x) \equiv \mathfrak{M}(v, x, u) = \begin{pmatrix} \mathfrak{I}(u, x, v) & \mathfrak{R}(u, x, v) \\ \mathfrak{R}(v, x, u) & \mathfrak{I}(v, x, u) \end{pmatrix}, \tag{iii}$$

which is the matrix operator of the standard invariant imbedding relation whose entries are the *complete* $\mathfrak{R}$ and $\mathfrak{I}$ operators. By "specific, concrete examples" is meant an actual construction of (i) and (ii) in, say, the plane-parallel case, where $N_+(y)$ may either be functions of direction on X_y or simply irradiances (fluxes per unit area which vary only with y and location in X_y). The result of such a construction, using the standard R and T operators of the classical theory, yields specific expressions for the extended $\mathfrak{R}$ and $\mathfrak{I}$ operators in (ii) which reduce to the complete operators in (iii) as indicated above. The details of this construction, while fundamentally simple and straightforward, are too long to be conveniently considered here.

Another special case of (i) which is of considerable interest results from the identifications: $w = x$ and $u = v$. Thus, (by using complete $\mathfrak{R}$ and $\mathfrak{I}$ operators) equation (i) reduces to

$$[N_+(x), N_-(x)] = [N_+(u), N_-(u)] \begin{pmatrix} \mathfrak{I}(u, x, u) & \mathfrak{R}(u, x, u) \\ \mathfrak{R}(u, x, u) & \mathfrak{I}(u, x, u) \end{pmatrix};$$

or, in view of the fact that $[N_+(x), N_-(x)]$ is simply $N(x)$, for arbitrary x, we see that

$$N(x) = N(u)\, \mathfrak{M}(u, x), \tag{iv}$$

where we have set

$$\mathfrak{M}(u, x; u, x) \equiv \mathfrak{M}(u, x, u) \equiv \mathfrak{M}(u, x).$$

Form (iv) of the generalized invariant imbedding relation yields the novel method of solution of transfer problems alluded to in the Introduction. The details of this method will be given in a subsequent study. For the present, we go on to consider the group property of the generalized operator $\mathfrak{M}$.

4. *Group Property.*—The standard $\mathfrak{M}$-operator given in the earlier form of the invariant imbedding relation[1] does not possess the group property because its range (in the function-theoretic sense) is too small. By extending the range of the $\mathfrak{M}$-operator to include the set $\mathfrak{N}$ (previously it was only the subset of ordered pairs $[N_+(x), N_-(x)]$, $a \leqslant x \leqslant b$) and by invoking the unicity condition (i.e., uniqueness

of $N(y)$ on X_y under given boundary conditions on X_a and X_b), the group property is then possessed by the generalized $\mathfrak{M}$-operator. This fact may already be seen in the special form (iv) of the generalized relation. To see this, let x, y, z be any three parameters in [a, b]. Then under the applications of (iv):

$$N(y) = N(x)\ \mathfrak{M}(x, y),$$

$$N(z) = N(y)\ \mathfrak{M}(y, z),$$

$$N(z) = N(x)\ \mathfrak{M}(x, z),$$

and using the unicity condition, we deduce that

$$\mathfrak{M}(x, z) = \mathfrak{M}(x, y)\ \mathfrak{M}(y, z).$$

Hence, the set $\Gamma_2(\mathfrak{M}) = \{\mathfrak{M}(x, y):\ (x, y) \epsilon [a, b] \times [a, b]\}$ is closed under the binary operation of *operator composition*. Any operator of the form $\mathfrak{M}(x, x)$, $x \epsilon [a, b]$ is already an identity operator and is uniquely of the form

$$\mathfrak{M}(x, x) = \begin{pmatrix} I_+ O \\ O\ I_- \end{pmatrix} = I,$$

where $I_\pm$ are the identity operators on the subsets $\mathfrak{N}_+$ and $\mathfrak{N}_-$ of $\mathfrak{N}$ consisting of the restrictions N_+, N_-, respectively, of N. Associativity of the operator composition holds, and finally,

$$\mathfrak{M}(x, y)\ \mathfrak{M}(y, x) = I$$

for every element $(x, y) \epsilon [a, b] \times [a, b]$. Hence, the set $\Gamma_2(\mathfrak{M})$ is formally a partial *group*. The adjective "partial" is used in the same sense as it is in "partially" ordered sets. Thus, in the present case, the product $\mathfrak{M}(w, x)\ \mathfrak{M}(y, z)$ is defined if $x = y$.

In a similar manner, it may be shown that the set $\Gamma_4(\mathfrak{M}) = \{\mathfrak{M}(v, x; u, w): (u, v), (w, x) \epsilon [a, b] \times [a, b]\}$ is formally a *partial group* under operator composition.

5. *Generalized Principles of Invariance and Operator Relations.*—Just as the standard invariant imbedding relation gave rise to the principles of invariance by setting the parameters equal to certain values,[1] so does the present generalized relation yield a corresponding set of generalized principles of invariance. Thus, from (i),

$$N_+(w) = N_+(u)\ \mathfrak{I}(u, w; v, x) + N_-(v)\ \mathfrak{R}(v, w; u, x);$$

$$N_-(x) = N_+(u)\ \mathfrak{R}(u, x; v, w) + N_-(v)\ \mathfrak{I}(v, x; u, w).$$

By setting $w = x$, we obtain the principles of invariance on the complete operator level. By then following an additional procedure, as outlined in reference 1, the four classical invariance statements are obtained from the complete-operator statements. Furthermore, the generalized functional equations governing the extended $\mathfrak{R}$ and $\mathfrak{I}$ operators may be obtained by the procedure outlined in reference 5.

In addition to the generalized relations above, the group property for $\Gamma_4(\mathfrak{M})$ yields four operator statements of which the following is one:

$$\mathfrak{I}(u, y; v, z) = \mathfrak{I}(u, w; v, x)\ \mathfrak{I}(w, y; x, z) + \mathfrak{R}(u, x; v, w)\ \mathfrak{R}(x, y; w, z).$$

This is obtained by equating the elements in the first column and first row of the $\mathfrak{M}$-matrix with the corresponding element in its product representation obtained by combining any two commensurate $\mathfrak{M}$-matrices according to the group property.

6. *Ontogenetics.*—At first appearance, the generalized invariant imbedding relation may seem to have no foundation in the established principles of radiative and neutron transport theory. That is, it appears to have been assembled in an ad hoc manner by means of some examples and an intuitively based inductive leap from the classical principles of invariance. However, recent work in the theory of general radiative processes on arbitrary discrete spaces has provided a firm foundation for not only the discrete version of (i) but its most general counterpart in an arbitrary discrete space.[6] Furthermore, the basis for a rigorous derivation in either the discrete or continuous case is given in reference 7.

Thus, while not all details have as yet been worked out, there now exists a complete analytical basis for the development of the concept of invariance, as used in the radiative and neutron transport contexts, from the classical R and T operator level through the invariant imbedding relation level up to the generalized level discussed in this note.

[1] Preisendorfer, R. W., "Invariant Imbedding Relation for the Principles of Invariance," these PROCEEDINGS, **44**, 320–323 (1958).

[2] Bellman, R., and R. Kalaba, "On the Principle of Invariant Imbedding and Propagation through Inhomogeneous Media," these PROCEEDINGS, **42**, 629–632 (1956).

[3] Chandrasekhar, S., *Radiative Transfer* (Oxford University Press, 1950).

[4] Ambarzumian, V. A., "Diffuse Reflection of Light by a Foggy Medium," *Compt. rend. (Doklady) acad. sci. U.R.S.S.*, **38**, 229–232 (1943).

[5] Preisendorfer, R. W., "Functional Relations for the R and T Operators on Plane-Parallel Media," these PROCEEDINGS, **44**, 323–327 (1958).

[6] Preisendorfer, R. W., *Principles of Invariance on Discrete Spaces*, SIO Ref. 59-55, University of California, La Jolla, California (1959).

[7] Preisendorfer, R. W., *Axiomatic Basis for the Principles of Radiative Transfer Theory*, SIO Ref. 59-75, University of California, La Jolla, California (1959).

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*SPECIFIC HOMOGRAFT TOLERANCE IN LYMPHOID CELLS OF LONG-LIVED RADIATION CHIMERAS**

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Several lines of experimental evidence, in studies on long-lived radiation bone marrow chimeras, indicate a state of tolerance or altered reactivity to normal tissue homografts,[1–3] heterografts,[4] and transplantable tumors.[5, 6] Long-lived radiation chimeras may be defined as irradiated mice, bearing transplanted cells of genetically foreign bone marrow, which have survived well beyond the usual period of secondary disease.[7] The possible mechanisms involved in the establishment of such homograft tolerance are of evident theoretical and practical interest (cf. ref. 8), particularly in view of the recent formulation of Lederberg,[9] based on the clonal selection

theory of immunity,[10] and in light of the possible relationships among the phenomena of homograft tolerance in radiation chimeras, specific acquired tolerance (Medawar, Billingham, and associates[11]), and tolerance observed under certain circumstances of parabiotic union in mice.[12, 13]

The present report is concerned with delineating further the nature and specificity of the tolerant state of long-lived homologous bone marrow radiation chimeras. In particular, we have directed our attention to the following questions: (1) chimerism of the lymphoid tissue cells of the radiation chimera (are the lymphoid cells of donor origin only, or are both donor and host genotype cells present?); (2) immunological reactivity of lymphoid cells of the chimera; (3) demonstration that lymphoid cells of the chimera are specifically tolerant.

The experimental data to be presented indicate that cells of both donor and recipient genotype are present in the lymphoid tissues of these mice, show that these cells are immunologically reactive, and suggest a state of mutual homograft tolerance between the donor and host cells present.

Materials and Methods.—Radiation chimeras: Young adult male and female (C57L × A)F_1 mice (so-called LAF_1)—3 to 4 months of age—were X-irradiated in a single exposure to 870 r of 250 KVP X-radiation, a dose approximately 100 r above the LD_{100} for these mice. A few hours later they received an intravenous injection of C_3H mouse strain bone marrow cells (5×10^6 nucleated cells) suspended in Tyrode's solution containing penicillin, as described previously.[14] The time course of occurrence of secondary disease and the longevity of such bone-marrow-treated mice have been described elsewhere.[15] In the present experiments, chimeras surviving for a year or more following irradiation and marrow injection were employed as lymphoid tissue donors in the test systems designed to demonstrate the presence of donor type cells (cf. ref. 3) and to study immunological reactivity on the part of these tissues. The C_3H marrow donor strain is known to belong to the histocompatibility antigenic group H-2k; the corresponding alleles in LAF_1 mice are designated H-2a and H-2b.

Isoantigen test system for donor cells: This test for specific isoantigen has been described briefly in a previous report.[3] It is based on the observation[18] that when normal LAF_1 mice receive a single injection of C_3H isoantigen (in the form of spleen cells, for example), a state of immunity is elicited such that protection against lethal X-irradiation given 1 week later is now not afforded by the injection of C_3H marrow.

In the present experiments, the spleen of each chimera was tested for the presence of C_3H isoantigen, essentially as described previously,[3] except that 5 test LAF_1 mice were used for each chimera. The blood of each chimera was tested for the presence of host type (i.e., LAF_1) isoantigen, as follows: 0.2 ml of fresh heparinized blood from the chimera was injected intraperitoneally into normal C_3H mice; one week later, these were exposed to an LD_{100} of X rays (810 r for this strain) and given an intravenous injection of $7–8 \times 10^6$ marrow cells from normal LAF_1 donors. Death of the recipient C_3H mice by 21 days, under these conditions, is interpreted to mean that the chimera mouse blood contains nucleated cells of host (i.e., LAF_1) origin.

Reactivity test system: The test system is based on the previous finding[16, 17] that protection of lethally X-irradiated mice by injected bone marrow is annulled when lymphoid tissue cells homologous to the injected bone marrow are administered. This was interpreted as a homograft rejection of the marrow cells by the injected, immunologically reactive, lymphoid cells, resulting in death of the lethally irradiated recipient mice from bone marrow failure.

Typically, the test system was set up as follows: A group of LAF_1 mice were exposed to a lethal dose of X rays (870 r); they were then divided into 3 subgroups, and each subgroup received (about 1 hr post-irradiation) an intravenous injection of bone marrow cell suspension (5×10^6 nucleated cells per mouse) derived from C_3H, DBA/2, or LAF_1 marrow donors, respectively. Blood was taken from the vena cava of each radiation chimera under chloroform anesthesia and each sample tested for LAF_1 isoantigen. The spleen from each chimera was removed individually, homogenized in a glass Potter-type homogenizer, and the tissue suspension from

each spleen was made up to 20 ml with Tyrode's solution. One ml of this cell suspension was injected intraperitoneally into each of 5 mice in each of 3 irradiated subgroups (above) which had received the bone marrow injections. The spleen cell injections were made approximately 2 hr after the marrow was injected. The remainder of the chimera spleen cell suspension was used in the isoantigen test (above).

Mortality at 21 days was the criterion of effect in this test system. If the chimera lymphoid cells are immunologically competent, they should bring about the rejection of the marrow derived from the third mouse strain (DBA/2), resulting in death of the irradiated recipient mice; furthermore, if the chimera lymphoid cells are tolerant, as is implied from our earlier study showing donor and host-type lung homograft acceptance by such radiation chimeras,[3] then the injected C_3H marrow as well as LAF_1 marrow should not be rejected, resulting therefore in the survival of the recipient, irradiated mice.

General procedures: The mice were put on drinking water containing the antibiotic polymyxin B (1 gm per liter) plus "Biosol" (5 mg per liter) *ad libitum* 1 day prior to radiation exposure and for 7 to 9 days following irradiation as a procedure designed to prevent *Pseudomonas* type infections. They were housed 2 per cage and fed Purina Laboratory Chow.

Results.—Specific immunological reactivity of chimera lymphoid cells: The data on the reactivity test system, employing normal lymphoid cells of known genotype are summarized in Table 1. Injection of 8×10^5 lymph node cells from normal

TABLE 1

REACTIVITY TEST SYSTEM. REJECTION OF BONE MARROW TRANSPLANTS BY HOMOLOGOUS LYMPHOID CELLS

Source of marrow injected (Mouse strain)	Lymph Node Cells Injected		Mortality of irradiated Recipients at 21 days* (No./total)
	Number	Strain	
C_3H	8×10^5	LAF_1	10/10
C_3H	5×10^6	LAF_1	10/10
C_3H	None	—	0/5
LAF_1	1×10^6	C_3H	1/12
LAF_1	5.8×10^6	C_3H	11/12
LAF_1	None	—	1/9

* All recipients are LAF_1 mice.
X-radiation dose: 880 r.

LAF_1 mice caused the rejection of C_3H marrow cell transplants in irradiated LAF_1 mice. When the injected marrow was isologous to the LAF_1 host, a larger number of homologous lymph node cells (in this instance, 5.8×10^6 C_3H node cells) was required to produce this effect.

Turning now to the data in Table 2, it can be seen, first, that in all 8 chimeras

TABLE 2

SPECIFIC REACTIVITY OF CHIMERA LYMPHOID CELLS

Animal no.	Age (months)	Test or Isoantigens*		Reactivity of Chimera Lymphoid Cells versus Marrow Transplants† Mortality (No./total)		
		C_3H (in spleen)	LAF_1 (in blood)	C_3H	LAF_1	DBA/2
1	13 3/4	4/5	Not tested	1/5	0/5	5/5
2	"	5/5	Not tested	0/5	1/5	5/5
3	"	5/5	Not tested	0/5	0/5	5/5
4	"	5/5	Not tested	0/5	0/5	5/5
5	14	5/5	4/4	1/5	0/5	5/5
6	14	5/5	4/4	0/5	0/5	5/5
7	14	4/5	4/5	0/5	0/5	5/5
8	14	5/5	3/3	1/4	0/5	5/5
None‡	—	1/16	5/10	1/9	0/10	3/15

* Transplantation immunity isoantigens; number of mice dead/total. See text.
† Recipients are LAF_1 mice; radiation dose: 870 r. They received I.V. injection of bone marrow from either C_3H, LAF_1, or DBA/2 donors plus I.P. injection of chimera spleen.
‡ Bone marrow injection only.

investigated, the spleens gave positive reactions for the presence of C_3H (i.e., donor) cells, as transplantation immunity isoantigen. In the 4 mice tested, the blood gave a positive test for host type (LAF_1) transplantation immunity isoantigen. The injection of approximately 10×10^6 chimera spleen cells into the lethally X-irradiated LAF_1 mice brought about abrogation of the protective effect of DBA/2 strain bone marrow, a mouse strain carrying the H-2d histocompatibility allele, homologous to both donor and host cells of the chimera. On the other hand, the injection of chimera spleen did not annul the protective effect of either C_3H marrow or LAF_1 marrow. In other words, the chimera spleen cells were reactive against the marrow cells of a third strain (DBA/2), but *not* against bone marrow cells of donor (C_3H) or of host (LAF_1) genotype. The conclusions seem warranted, therefore, that these long-term survivors, following LD_{100} X-radiation exposure and administration of homologus (C_3H strain) bone marrow, are still in the chimeric state after 14 months, that their spleens contain cells capable of immunological reactivity (i.e., rejection of DBA/2 bone marrow cell transplants), and that these spleen cells are at the same time nonreactive, i.e., tolerant, with respect to transplants of C_3H and LAF_1 genotype marrow cells.

Donor type immunologically reactive cells in long-lived radiation chimeras: The next series of experiments were designed to obtain evidence for the presence of immunologically competent cells of donor origin (C_3H genotype) in lymphoid tissues of the chimeras. Two types of designs were used. In the first, ($C_3H \times DBA/2)F_1$ hybrid mice, previously sensitized by injection with LAF_1 isoantigen (as spleen cells) were exposed to a lethal dose of X-radiation (880 r), then given a marrow cell transplant (I.V. injection of 5×10^6 BALB/c marrow cells). These mice then received an injection of spleen cells from the radiation chimeras. The results are summarized in Table 3. Under the conditions of pre-immunization

TABLE 3

REJECTION OF HOMOLOGOUS MARROW CELL TRANSPLANTS BY IMMUNOLOGICALLY REACTIVE CELLS OF DONOR ORIGIN IN SPLEENS OF RADIATION CHIMERAS

Spleen Cells Injected		21-Day mortality of irradiated ($C_3H \times DBA/2)F_1$ recipients*
Source	No. of cells	(No./total)
Chimera No. 13	5×10^6	5/5
Chimera No. 14	5×10^6	5/5
Chimera No. 15	4×10^6	4/5
Chimera No. 16	5×10^6	5/5
Normal C_3H	4×10^6	9/10
Normal LAF_1	5×10^6	1/10

* These recipients had been sensitized (anti-LAF_1) one week prior to X-irradiation (880 r), by a single intraperitoneal injection of LAF_1 spleen cells, as homogenate. A few hours after irradiation, they received a single I.V. injection of 5×10^6 bone marrow cells from normal BALB/c mice, followed by I.P. injection of the spleen cells.

of the ($C_3H \times DBA/2)F_1$ recipient mice (anti-LAF_1), it can be seen that the reactivity of the injected normal LAF_1 spleen cells is prevented, i.e., the BALB/c marrow "takes." By contrast, the injection of normal C_3H spleen cells under these conditions annuls the "take" of BALB/c bone marrow. (This procedure of pre-immunization has been utilized by Siskind *et al.*[28] to prevent or minimize the occurrence of "runt disease" following the injection of homologous lymphoid cells into newborn mice.) Now, when cells from the late chimeras were injected under these conditions, the BALB/c marrow cells were rejected as inferred from the

21-day mortality of the lethally X-irradiated recipients. These results, then, suggest the presence of C_3H type immunologically reactive cells in the spleens of the radiation chimeras.

The second approach to the same question involved the investigation of the capacity of spleen cells from the chimeras to produce the lethal "wasting syndrome" when injected into sublethally irradiated $(C_3H \times DBA/2)F_1$ hybrid mice. If the spleen of the chimeras contain immunologically reactive C_3H cells, then these cells would bear the relationship of parental strain to the $(C_3H \times DBA/2)F_1$ hybrids and evoke this wasting syndrome, as it occurs in other parental-F_1 hybrid combinations.[23] The experimental results are given in Table 4. A lethal syndrome with characteristic wasting was elicited in all of the 500 r irradiated $(C_3H \times DBA/2)F_1$ recipients injected with chimera spleen cells. These data show that immunologically reactive C_3H type cells are, indeed, present in the spleens of the 4 long-lived radiation chimeras examined.

TABLE 4

PRODUCTION OF LETHAL WASTING SYNDROME IN SUBLETHALLY X-IRRADIATED $(C_3H \times DBA/2)F_1$ HYBRIDS BY SPLEEN CELLS FROM RADIATION CHIMERAS

Spleen Cell Injected		21-Day mortality of 500 r X-irradiated $(C_3H \times DBA/2)F_1$ recipients (No./total)
Source	No. of cells	
Chimera No. 13	19×10^6	10/10
Chimera No. 14	18×10^6	10/10
Chimera No. 15	15×10^6	10/10
Chimera No. 16	25×10^6	10/10
Normal C_3H	16×10^6	10/10
Normal LAF_1	20×10^6	0/10

Minimal occurrence of secondary disease in irradiated LAF_1 mice injected with C_3H marrow plus chimera spleen: The occurrence of secondary disease is minimal or absent among certain strains of mice when fetal or newborn homologous hematopoietic tissue cells are injected into lethally X-irradiated recipients.[19, 20] This is considered to be a consequence of the fact that fetal or newborn hematopoietic tissues contain immunologically nonreactive and immature lymphoid cell elements, which become tolerant to the isoantigens of the homologous host as they mature. Therefore, no graft-versus-host reaction occurs, and consequently no secondary disease syndrome develops.

In this connection, therefore, it was of interest to ascertain whether injection of spleen cells from the radiation chimeras would modify the occurrence of secondary disease. A group of LAF_1 mice was X-irradiated with 880 r and injected with 3×10^6 C_3H strain marrow cells per mouse. A few hours later, subgroups of 3 to 5 mice from this population received an intraperitoneal injection of spleen cells from each of 8 long-lived radiation chimeras (approximately 14 months post-irradiation). The data are summarized in Table 5. It is evident that the injection of chimera spleen cells, together with normal C_3H bone marrow, greatly reduces the occurrence of secondary disease deaths in this population by 5 months post-radiation. In 4 of the subgroups receiving chimera spleen plus normal C_3H marrow, no deaths occurred, and in the other 4 subgroups, only sporadic late deaths were observed. By contrast, from our previous experience with a large number of lethally X-irradiated LAF_1 mice injected with C_3H marrow, secondary

TABLE 5

MINIMAL OCCURRENCE OF SECONDARY DISEASE IN IRRADIATED LAF_1 MICE RECEIVING C_3H MARROW PLUS CHIMERA SPLEEN

Material Injected		Mortality at 5 Months	
C_3H marrow cells (no.)	Chimera spleen cells (no.)	No./total	Time (days)
3×10^6	11×10^6	0/5	—
3×10^6	12×10^6	0/4	—
3×10^6	16×10^6	1/4	150
3×10^6	5×10^6	1/5	23
3×10^5	17×10^6	1/5	19
3×10^6	16×10^6	1/5	49
3×10^5	11×10^6	0/5	—
3×10^6	13×10^6	0/3	—
None	None	13/13	11.5*

Recipients are LAF_1 mice; radiation dose: 880 r.
* Mean time of death. Range was 9 to 14 days.

disease accounts for deaths in about 60 per cent of the population by 5 months post-irradiation.[15] The injection of normal C_3H lymphoid tissue cells plus C_3H marrow into lethally irradiated LAF_1 mice is known to bring about a more severe and early secondary disease syndrome.[15] Therefore, the fact that in the present experiments the injection of chimera spleen cells greatly reduces the occurrence of secondary disease is again indicative that these cells are nonreactive with respect to the LAF_1 host. Presumably, these tolerant lymphoid cells have repopulated lymphoid sites of the irradiated recipients (cf. ref. 29).

Discussion.—The foregoing experimental data provide additional evidence for existence of a state of homograft tolerance in long-lived homologous radiation chimeras, i.e., those which have escaped or survived the secondary disease syndrome and have lived on for periods of a year or more post-irradiation. The lymphoid cells from these C_3H-LAF_1 radiation chimeras cause the rejection of marrow transplants derived from a mouse strain (DBA/2 or BALB/c) genetically foreign to both the donor and host cell genotype in the chimera itself. However, these chimera lymphoid cells are nonreactive, that is to say, tolerant, with respect to marrow cell transplants of C_3H or LAF_1 origin. These data, taken together with the fact of the presence of C_3H isoantigen, presumed to be C_3H cells (cf. ref. 3), in the lymph nodes and spleen, are tantamount to a demonstration of a state of specific tolerance in these chimeras.

What is the genetic identity of this specifically tolerant, and yet immunologically reactive, population of cells in the lymphoid tissues of these chimeras? The data in Tables 3 and 4 show that at least some of these reactive cells are of donor (i.e., C_3H) origin, derived from the initial bone marrow cell inoculum. The presence of host (i.e., LAF_1) cells also is *inferred* from the fact that the peripheral blood of the chimeras tested gave a positive test for LAF_1 transplantation immunity isoantigens (see Table 2). This could be due to the lymphocytes or to the granulocytes of host origin present in the peripheral blood. Assuming from this that recovery of host lymphopoietic activity has occurred, it may be suggested that a state of mutual tolerance exists between donor and host type lymphoid cells in the long-lived radiation chimeras, in addition to the condition of immunological reactivity in the chimera, leading to rejection of cells of a third mouse strain. Unpublished observations (Davis and Cole) show that these late chimeras will accept tail skin homografts from normal C_3H or LAF_1 donors but regularly reject BALB/c

or DBA/2 skin homograft. However, it should be pointed out in this connection that direct experimental evidence for the presence of immunologically competent cells of host origin in these long-lived chimeras is not yet available.

If the above conclusions be valid, it is pertinent next to consider possible mechanisms by which homograft tolerance may have been produced in these chimeras and the related question of the connection between these findings and that of the phenomenon of acquired tolerance in animals treated with genetically foreign cells at birth.[11] In this context, it has been proposed by Barnes and Loutit[5] and by Barnes *et al.* (1) that skin homograft tolerance in their long-lived radiation chimeras results from "actively acquired tolerance to donor type cells in the sense of Billingham, Brent, and Medawar."[11] Such a view was also put forward by Trentin.[21]

Let us now briefly consider this concept of tolerance in late chimeras in light of the present findings and viewed from the standpoint of the Lederberg formulation.[9] One can readily assume that with supralethal doses of X-radiation (such as used here) all the mature, potential antibody-producing cells, i.e., those not yet sensitized with specific isoantigen, are destroyed. With the administration of genetically foreign isoantigen (in the form of homologous bone marrow cells) immediately after irradiation, we have a situation possibly quite analogous to that of the newborn recipient mouse injected with homologous cells. In the latter instance, there are no mature host cells present, specific for the homologous antigen—cells which are present in the nonirradiated adult animal and which give rise to the immune state. If in time the host lymphoid cells in the radiation chimera can begin to regenerate, they will arise from immature ("hypersensitive") cells in the presence of homologous donor isoantigen, so that any cells potentially specific for that isoantigen will be killed off.[9] This would account for a specific tolerance on the part of the host cells in the chimera, a tolerance which would continue so long as the donor type isoantigen (in this case in the form of dividing cells) persists. In this connection the experimental data of Mitchison[22] and of Nossal[26] further emphasize the necessity of persistence of the foreign antigen for maintenance of tolerance.

An analogous argument to the above would be invoked in the attempt to account for homograft tolerance on the part of *donor cells for host isoantigen* in the late chimera (as proposed here), wherein the original donor cells injected were obtained from mature animals. In this instance, one would have to postulate first that the mature, immunologically competent cells in the marrow inoculum and in the proliferating donor cell population in the irradiated host become functionally inactivated or would die in the course of their continuous and prolonged reactivity against host isoantigen. (It is to be noted that we are speaking here of chimeras which survive the secondary disease syndrome.) Such a concept has been proposed in connection with the parental F_1 hybrid lymphoid cell chimera studies of the authors.[23] In this case, then, inactivation or death of all the mature donor lymphoid cells would lead again to a condition in which only immature cells remain. These would then be subject, in the presence of host isoantigen, to the same clonal selection[9] discussed above for the host cells, resulting in a specifically tolerant cell population of donor origin.

On the basis of these considerations, it may be suggested that a similar state of

tolerance on the part of *donor* cells for host isoantigen exists in the case of actively acquired homograft tolerance following injection of homologous cells into newborn recipients. This view is consistent also with the findings of Rubin[12] and of Martinez *et al.*[13] that prolonged parabiosis between adult parental strain and adult F_1 hybrid mice (both nonirradiated) leads to a state of homograft tolerance on the part of the parental strain mice to F_1 hybrid skin grafts among the surviving parabionts. It is of interest in this context that Simonsen[30] has recently provided evidence suggesting the presence of specifically tolerant donor cells in spleens of mice with runt disease. In this case, runting was produced by the injection of adult C_3H spleen cells into infant $(C_3H \times ST/A)F_1$ hybrids. Thus, development of the state of tolerance in a *nonirradiated, initially adult* lymphoid cell population appears to be possible.

To what extent the present formulation of specific homograft tolerance in long-lived radiation chimeras is critically distinguishable in operational terms from the notion of immunologic tolerance, as a consequence of immunological paralysis in the sense of Felton,[24] we are not as yet in the position to state (cf. ref. 25). However, the observation by Martinez *et al.*[27] that acquired immunological tolerance of skin homografts can be transferred to adult nonirradiated mice by parabiotic union would seem difficult to interpret in terms of immunological paralysis. Experiments aimed at resolving this question further are planned.

Summary.—$(L \times A)F_1$ hybrid mice were exposed to an LD_{100} of X-rays (870 r) and were then injected intravenously with bone marrow cells from genetically foreign (i.e., homologous) normal C_3H strain mice. It was found that the long-term survivors (14 months post-irradiation) were still chimeras, as evidenced by the presence in the spleen of transplantation immunity isoantigens specific for the marrow donor strain (C_3H). Immunological reactivity of lymphoid tissue cells from these chimeras was assessed by their capacity, when transferred to lethally X-irradiated $(L \times A)F_1$ mice, to cause the rejection of homologous bone marrow. These chimera lymphoid cells brought about the rejection of marrow transplants derived from normal DBA/2 or BALB/c mice-strains which are genetically foreign with respect to both the donor and host genotype of the chimeras. However, lymphoid cells from the chimeras were found to be nonreactive, i.e., tolerant, towards marrow transplants of $(L \times A)F_1$ or of C_3H origin. Data are presented which indicate strongly the presence of immunologically reactive cells of donor (C_3H) genotype in the lymphoid tissues of these long-lived chimeras. It is suggested that a state of mutual homograft tolerance exists between the donor and host lymphoid cells in these long-lived radiation chimeras, while at the same time, these cells are able to reject grafts of cells which are homologous to both donor and host type. The implications of these findings as they relate to the phenomenon of actively acquired tolerance and to the clonal selection theory of immunity are discussed.

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THE GEOMETRY OF COILING IN GASTROPODS

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The geometrical form of coiled invertebrate shells has long attracted the attentions of zoologists and mathematicians. Coiling is exhibited, to varying degrees, in such diverse groups as the Brachiopoda, Foraminifera, and Mollusca. Most of the work in this field has been directed, however, at the gastropods and coiled cephalopods. Among the early references to the geometry of gastropods are the studies of Réamur,[1] Mosely,[2, 3] Naumann,[4] and Blake.[5] These and other works of the same period have been ably digested and summarized by D'Arcy Thompson.[6]

The principal thesis of Thompson's work is that growth in coiled forms follows

rather rigid mathematical laws. According to Thompson, a fundamental characteristic of the coiled shell is that, as growth proceeds, "... the curve traced in space is, in all cases, an equiangular [logarithmic] spiral ..."[7] Many of the basic differences between species are ascribed to differences in the angle of this spiral. Additional characteristics considered to be fundamental were presented as follows: (1) the apical angle (the vertex angle of a cone enveloping the spire of the snail), (2) the shape of the generating curve (which may be thought of, in most instances, as the whorl cross section), and (3) the angle of retardation (expressing the rate of growth of the inner as compared with the outer part of each whorl).[8] The measurement of these three parameters and of the angle of the equiangular spiral should, in Thompson's view, suffice to describe the basic form of any coiled gastropod.

Thompson's scheme for describing the gastropod shell does not consider such morphologic features as ornamentation, growth lines, or the form of the protoconch: it is intended only to include the basic shape upon which the complete morphology is built. As such, it is an idea which should be exceedingly attractive to the zoologist and paleontologist. From the viewpoint of pure description, it represents an attempt to diagnose shell form in terms of truly natural parameters, related to growth, rather than in terms of rather arbitrary characters such as width of body whorl or spire height.

Beyond simple description, the possibilities for fruitful application of this kind of approach to the study of gastropod evolution are manifold. But Thompson's generalizations have not been widely used by the zoologists and paleontologists actively working with problems of comparative morphology in gastropods. This is to be expected in that some of the basic parameters (the angle of retardation, in particular) are difficult or impossible to measure in most specimen material. Furthermore, there appear to be several basic fallacies in Thompson's reasoning. For example, the measured value of the apical angle is very sensitive to minor changes in configuration of the generating curve, or whorl cross section. Such changes often do not affect the basic form of the shell but do have a profound effect on Thompson's diagnosis of its geometry because the apical angle is one of his basic parameters and is also used in the calculation of the angle of the equiangular spiral. Another example involves the use of the "sutural angle," the angle formed by the axis of coiling and the projection (in the plane of the axis) of a suture between two successive whorls. This angle is used in Thompson's calculations and is assumed to be constant for a given snail. This assumption is invalid, however, because as the whorl enlarges, the sutures must diverge, bringing about a progressive change in the sutural angle: this can be observed readily in many specimens.

In the literature since Thompson, there are several noteworthy papers on the geometry of coiling but these studies represent only extensions of Thompson's work and have the same shortcomings. There still remains a paucity of application of geometric characteristics to practical problems of gastropod systematics and evolution.

In the present paper, a fresh approach to the general problem of morphologic description of coiled gastropods is considered. The objectives are practical rather than theoretical, and the descriptive scheme which is proposed should be evaluated on the basis of its direct applicability to gastropod morphology, fossil and Recent.

The Nature of the Coiled Shell.—Perhaps Thompson's most significant contribution to gastropod geometry was to emphasize a few characteristics of shell form which may be built upon in the formulation of a workable analytical scheme. For the present purposes, the most important of these are as follows:

1. *In a single individual, the shape of the generating curve remains constant throughout growth.* With relatively few exceptions, the shape of the whorl cross section remains unchanged during the development of the snail (not considering the protoconch). As a result, the body whorl of a young individual has the same shape as that of an older individual except where a pronounced lip is formed in maturity.

2. *In a single individual, the rate of expansion of the generating curve is approximately constant.* If a series of whorl widths is measured at regular intervals on a single specimen, some variation in the ratios of successive widths is observed; the variation may even be correlated with growth. But at the species and genus level, such minor variations cease to be significant, and the average ratio (defined as w, where successive measurements are separated by one full revolution about the axis) may be used to contrast differing basic shell forms.

3. *In a single individual, the relative amount of overlap of one whorl on the preceding one is approximately constant.* In some forms, such as *Conus*, a given whorl almost completely overlaps the previous one, as measured in a direction parallel to the axis of coiling. Near the other extreme, there is almost no overlap in the height dimension of whorls of *Epitonium*. For any individual, however, the overlap is nearly constant.

4. *In a single individual, a constant ratio relates size of generating curve to the distance between the axis of coiling and a given point on the generating curve.* The axis is considered as a straight line which bisects the apical angle. In some forms, the whorl is never in contact with the axis of coiling: these are the umbilicate gastropods. More generally, however, the whorl is in contact with the axis or overlaps it slightly. In each case, the relation between whorl and axis remains constant throughout growth.

The four generalizations presented above simply reflect the fact that there is rarely much variation in coiling characteristics during growth of an individual gastropod. A young form has a shell which is essentially a scaled-down replica of the shell of an older form. However, the several characteristics show marked variation from genus to genus. Often, the differences between two quite distinct morphological types are caused by variation in only one of these characteristics.

As an example, the effects of changing only the amount of overlap of successive whorls are shown in a series of hypothetical gastropods in Figure 1. In this figure, the shape of the generating curve, the rate of expansion of this curve, and the distance from the curve to the axis (relative to the size of the curve) have been held constant; only the amount of overlap has been changed. The effects of increasing overlap are (1) increase in apical angle, (2) change in the shape of the aperture, and (3) marked decrease in the length-width ratio of the whole shell; in short, the whole morphologic aspect is changed simply by altering the amount of whorl overlap.

At this point, it is important to make a distinction between the shape of the generating curve and the shape of the aperture. Thompson defined the generating curve as a closed curve which increases its dimensions continuously as it revolves

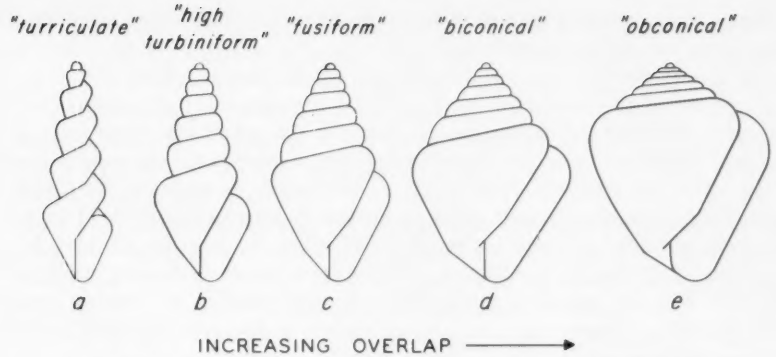

FIG. 1.—Effects on gastropod morphology of changes in amount of whorl overlap.

about a fixed axis (the axis of coiling). In forms such as the one in Figure 1*a*, the shape of this curve is observable: it is nearly equivalent to the aperture or to a cross section of any whorl. But as the amount of whorl overlap increases, part of the outline of the generating curve becomes obscure. In fact, the gastropod represented in Figure 1*e* does not utilize the entire generating curve; rather, only the outer part is actually deposited by the organism. The aperture in this case is enclosed between the inner surface of the body whorl and the outer surface of the previous whorl and reflects the generating curve only along its outer margin.

In Figure 2, the effects of changing the rate of whorl expansion are shown by

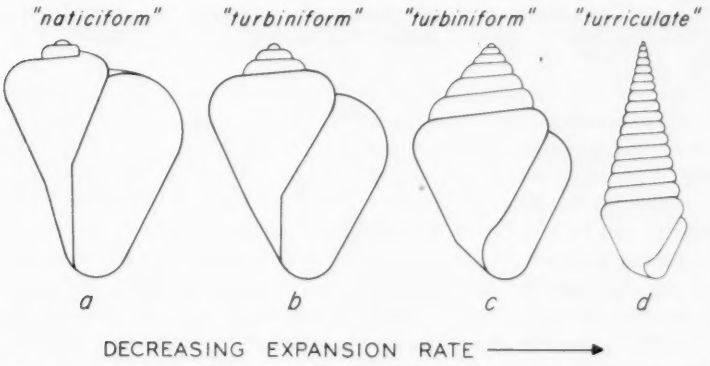

FIG. 2.—Effects on gastropod morphology of changes in rate of whorl expansion.

another series of hypothetical gastropods. As in the previous case, all other coiling characteristics have been held constant. In Figure 2*a*, one complete revolution of the generating curve causes a 300 per cent increase in the dimensions of the generating curve; the increase per revolution is only 10 per cent in Figure 2*d*. Here again, the change in one fundamental characteristic of the shell produces a marked change in over-all appearance. Note also, by comparing Figures 1 and

2, that a given value of the apical angle can be produced by more than one combination of the basic parameters.

The Description of Gastropod Morphology.—It is implied, above, that the basic coiling form of the gastropod can be defined through only four parameters (not the same four that Thompson used): shape of the generating curve, rate of expansion of this curve, amount of overlap between successive whorls, and the position of the whorl relative to the axis. This possibility may be tested by describing the appropriate characteristics on actual specimens and then attempting to reproduce the form of each specimen by purely mechanical means. An example of this procedure is illustrated in Figure 3. The species chosen is *Ecphora quadricostata* (Say), of Miocene age (and, incidentally, the first fossil ever described from the New World[9]). Figure 3*a* is an artist's sketch of the specimen and Figure 3*b* represents the scheme here proposed to describe coiling form.

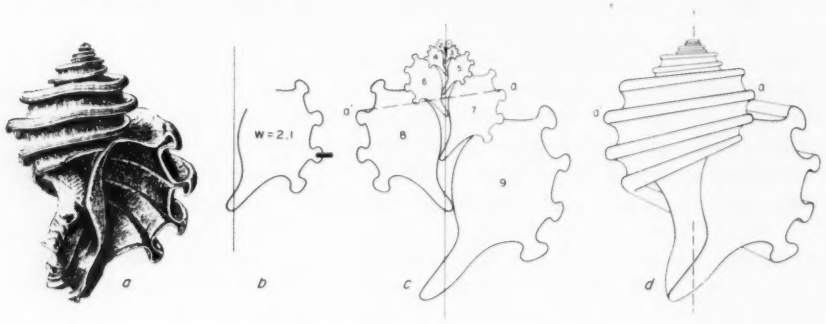

FIG. 3.—Reconstruction of basic form of gastropod from its coiling characteristics. 3*a*, drawing of *Ecphora quadricostata*[10]; 3*b*, basic coiling characteristics; 3*c*, *d*, reconstruction of morphology from 3*b*.

In Figure 3*b*, the vertical line indicates the axis of coiling, that is, the axis about which the generating curve revolves. The outline to the right of this axis is that portion of the generating curve which may be observed in the species, and the position of the curve relative to the axis is shown. The tick-mark on the generating curve represents the point of contact with the succeeding whorl (which would be the suture) and thus indicates the amount of overlap. Finally, the rate of whorl expansion (w) is shown by a number which indicates the ratio between *any* dimension of one whorl and the same dimension of the preceding whorl (the two measurements being separated by 360 degrees of rotation.) In this case, the dimension used was the distance between two of the prominent ribs on the generating curve, but any other convenient dimension could have been used. (In most species, the distance between successive sutures along a line through the apex of the shell proves to be the most favorable dimension.) All characteristics shown in Figure 3*b*, except the value of w, were obtained with a camera lucida.

The remaining sketches in Figure 3 show the procedure for reproducing *Ecphora*. First, an axis of arbitrary length is drawn. Next, replicas of the generating curve are drawn in varying sizes by using the lens and ground glass assembly of a portrait camera. The size of the first (and smallest) generating curve is purely arbitrary, and sizes of succeeding copies are determined by the expansion rate (w). The

size (x) of the generating curve is related to the number of revolutions (y) about the axis in the following manner: $x = w^y$. The size of the generating curve which is second from the top on the right-hand side of the axis (#3 in Fig. 3*c*) is w times the size of the preceding generating curve on the same side (#1), #5 is w times #3, and so on. Following the same relation, a given generating curve on the left-hand side of the axis (#2, for example) is larger than the preceding one on the *right* (#1) by a factor of the square root of w.

The series of generating curves may then be distributed along the axis in such a way that they maintain a constant distance from the axis (relative to their respective sizes) and that their vertical position reproduces the amount of overlap indicated by the tick-mark on the outline of the generating curve.

The result of the above (Fig. 3*c*) is essentially a cross section of *Ecphora*. A perspective drawing (Fig. 3*d*) may be constructed readily from this cross section by connecting like points (such as a and a') on outlines of the generating curve which are 180 degrees apart.

It should be emphasized that the complete reconstruction of the gastropod form (Fig. 3*c*, *d*) serves only to demonstrate the validity of the system. Figure 3*b* suffices to describe *E. quadricostata*.

The basic description of a wider variety of gastropod forms is shown in Figure 4. In forms like *Conus*, only a part of the generating curve may actually be observed,

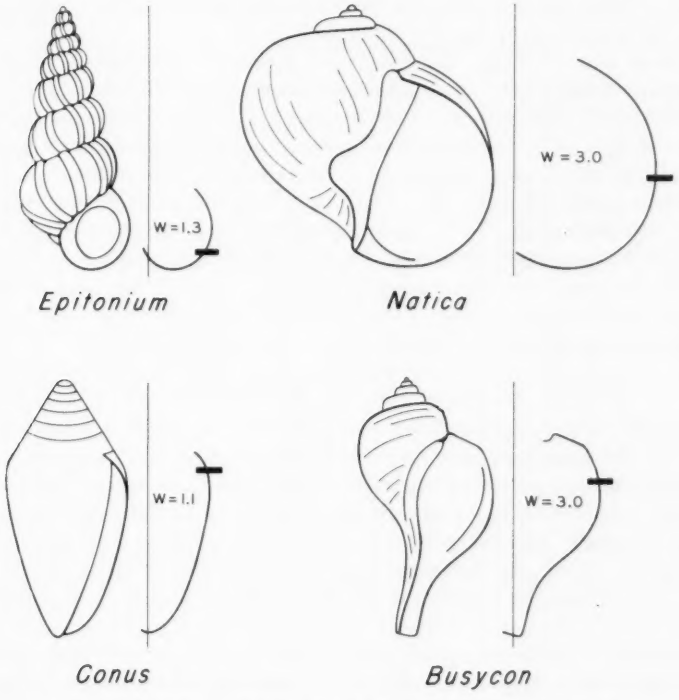

FIG. 4.—Basic coiling characteristics of several common gastropods.

but this does not detract from the feasibility of description of the basic geometry. Also, features of ornamentation such as are commonly observed in *Epitonium* would not be found in the reproduction of its coiling form.

It is concluded from the above that the basic description of morphology (excluding ornamentation, etc.) can be accomplished with one calculated ratio (w) and a line drawing showing the other critical elements (as in Fig. 3*b*). Four parameters are used, and it should be noted that the shape of the generating curve is the only one which has been carried over from Thompson's list of four fundamental characteristics.

Applications.—It is not suggested that the proposed scheme should replace conventional procedures in morphologic description. Only the basic coiling form is encompassed by the method. This basic form should be expressed by as simple and consistent a scheme as possible, however, and thus the present proposals may lead to more efficiency in description. Other areas of possible application are as follows:

Ontogenetic studies: Variation in coiling form during growth has long been known. This variation represents departure from the generalizations set forth above; striking examples are those species which have coeloconoid shells, that is, those which exhibit a concave spire in lateral profile. It can be shown (through series of measurements) that ontogenetic variation in gastropod coiling is explicable in terms of systematic and regular changes in one or more of the four basic parameters. The definition and measurement of these parameters provide a convenient tool with which to study this variation.

Phylogeny and evolution: Figures 1 and 2 suggest that spectra of gradation exist between morphologic types which are generally distinguished by terms such as "turriculate," "turbiniform," and "biconical." The recognition of gradational series in zoological or paleontological material would be much more readily accomplished by description in terms of the basic parameters of coiling. Evolutionary series may thus be recognized which have been obscured by the conventional methods of description. A fundamental question here is whether the four parameters that have been defined have genetic reality and would thus be expected to change independently and in a regular fashion. This question can be answered only after more is learned from studies of gastropod genetics and work with comparative morphology in evolving lineages.

Ecology: It is tempting to relate coiling form to the gastropod environment. It will be noted, for example, in Figure 2, that the surface area of shell material deposited by the organism relative to the volume of the shell bears an inverse relation to the whorl expansion rate. This suggests that an increase in expansion rate tends toward the conservation of calcium carbonate. Because of this, the geographic distribution of certain combinations of coiling characteristics might be related to ecologic factors bearing on carbonate metabolism, such as temperature and salinity. There is, indeed, a suggestion of such a correlation with water temperature in western Atlantic marine snails, but further study is necessary for confirmation.

Conclusions.—As stated in the introduction, the main objective of the present study is to devise a system of description which may be applied readily to a wide range of gastropod types. For this reason, the treatment lacks mathematical

elegance. Although much more precision could be added to the system, it is doubtful whether the purely qualitative aspects could ever be removed. The shape of the generating curve, for example, is generally irregular enough to greatly hinder mathematical description. Furthermore, the complete generating curve, as a closed curve, can rarely be seen, and thus its position relative to the axis of coiling is difficult to define mathematically. Finally, the departures from regularity observed in ontogeny discourage completely rigorous analysis of gastropod geometry.

The author is greatly indebted to the following persons for contributing valuable advice and criticism at various stages of this study: Ernst Cloos, J. D. H. Donnay, Hans P. Eugster, F. J. Pettijohn, Susan S. Raup, and the members of the Paleontological Society of Washington. He also wishes to thank John S. Spurbeck, who prepared the illustrations, and Mary Gill, who typed the manuscript.

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[2] Mosely, H., *Trans. Roy. Soc. (London)*, part 1, 351–370 (1838).

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[6] Thompson, D'A., *On Growth and Form* (New York: Macmillan Co., 1942), pp. 748–849.

[7] Thompson, *ibid.*, p. 812. The defining characteristic of this spiral (as a plane figure) is that the angle formed by a tangent to the spiral and a radius (at the point of tangency) is constant. The size of the constant angle controls, therefore, the shape of the spiral.

[8] The reader is referred to Thompson (*ibid.*, pp. 798–805) for a fuller discussion of the angle of retardation.

[9] Lister, M., *Historia sive synopsis methodicae Conchyliorum* (London, 1685), Plate 1059, Figure 2.

[10] Martin, G. C., *Maryland Geological Survey*, Miocene Volume, Plate 52, Figure 1 (1904).

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*PROLONGATION OF THE LIFE SPAN OF KOKANEE SALMON (ONCORHYNCHUS NERKA KENNERLYI) BY CASTRATION BEFORE BEGINNING OF GONAD DEVELOPMENT**

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The present investigation was undertaken as an initial approach to the problem of the nature of the post-spawning death of Pacific Coast salmon. The regularity with which all five species of this salmon die after their first reproduction, no matter whether the normal life cycle of the species is 2, 3, or 4 years, strongly suggests a causal relationship between the spawning act and subsequent death. However, it is also possible that the life span of each species is biologically fixed and that spawning constitutes the final phase. Would prevention of spawning extend the salmon's life cycle? An attempt to answer this question was made by castrating a number of sexually immature kokanee and observing them over a period of years. Unoperated controls of the same hatch were maintained under the same environmental conditions.

Since the beginning of this experiment in 1954 much has been learned about the changes that take place in migrating and spawning salmon. The sexually mature fish exhibits extensive histological alterations in its organs and tissues, degenerations and hyperplasias[1-3] accompanied by marked physiological disturbances,[4] most significant of which is a greatly increased concentration of 17-hydroxycorticosteroids (17-OHCS) in the blood.[5] This is associated with pronounced hyperplasia of the adrenal cortical tissue.[2] These physiological and histological changes begin during the spawning migration some time before full sexual maturation. It seems probable that the catabolic action of high levels of plasma 17-OHCS over prolonged periods, combined with the starved state of the migrating and spawning salmon, could cause the deterioration present at the termination of the life cycle. Yet lacking information as to whether or not changes of a similar nature would take place in the absence of sexual maturation this explanation until now has been entirely presumptive. The results of the present study, on which three brief preliminary reports have already been published,[6, 7] show that castrated kokanee salmon do not deteriorate and die at the time at which the life cycle of this species normally ends.

Materials and Methods.—Kokanee: The kokanee, a variant of the blue back (sockeye) salmon (*O. nerka nerka*), has become landlocked, lives in lakes and spawns either in tributary streams or in the lake itself. The kokanee's normal life cycle is the same as that of its kindred form *nerka nerka;* namely, 4 years. It is a convenient experimental salmon not only because it lives in fresh water but also because of its small size: the full grown adults of most well established populations seldom reach more than 15 to 16 in. in length.

The kokanee employed in the present experiment were hatched at the Lake Tahoe fish hatchery of the California Department of Fish and Game from eggs obtained at Park Fork, Idaho. Hatching was completed by April 19, 1952. Until a year of age the fish were held in a wooden trough approximately 15 × 4 × 4 ft., supplied by an abundant flow of spring water with winter Fahrenheit temperatures in the low 40's and summer temperatures in the mid 40's to mid 50's. Water was kept at a depth of 2 ft. They were then transferred to a small dirt pond about 25 × 10 ft. with the same supply of water at a depth of about 18 in. A wooden platform shelter 4 × 4 ft. was placed at the intake end of the pond. During the winter deep snow not infrequently made the pond inaccessible for considerable periods of time. The kokanee were fed a mixture of liver and seafish. During the last three years of the experiment at Crystal Lake, where they had been transferred as will be described later, they were given mostly seafish-rock cod. How much of this diet the kokanee ate is not known since they apparently preferred snails brought in by the water supply.

Castration: In May 1954, at the age of 2 years 1 month, 175 kokanee 6–7 in. long were castrated. The details of the technique employed have been described in a former publication.[8] Briefly, the fish were anesthetized in a 1:20,000 dilution of tricaine methanesulphonate (MS222-Sandoz) and then placed on an operating board with the head submerged in a 1:25,000 dilution of the same anesthetic. The gonads were removed through a midline abdominal incision, the attempt being made to excise the cephalic pole completely and as much of the posterior portion of the gonad as possible by pulling the gonad taut and cutting it off well down toward the caudal attachment. A few milligrams of penicillin[9] were placed in the peritoneal cavity before closing the wound with absorbable chromic gut. Average weights of testes were 59 mg, and of the ovaries, 80 mg. Histological examination showed them to be in the infantile stage.

Males were marked by clipping the right pelvic and adipose fins; females, by clipping the left pelvic and adipose fins. The operated kokanee were kept in the hatchery for 3 weeks in covered troughs to permit healing of the incision before transferring to the pond, which also contained 305 control unoperated fish.

Because of the distance of the Lake Tahoe hatchery, and later the Crystal Lake hatchery, from Stanford, population counts, quantitative observations on growth, photography, and the like

could be made only at infrequent intervals. However, the hatchery personnel took unusual interest in these experimental kokanee and inspected them twice a day or more frequently in the fall when the controls were maturing and dying. They performed autopsies on all dead fish, recording length, changes in morphology and skin, presence and state of gonads, size of eggs, and other pertinent data.[10] The attempt was made to secure fish immediately after death, or if possible in a moribund state in order to obtain tissues satisfactory for histological study. Postmortem fish tissues deteriorate rapidly.

The live fish were handled as little and as infrequently as possible since kokanee seem to be more sensitive to trauma than other hatchery salmonids and are distinctly more difficult to rear in captivity.

For weighing, measuring and photographing, the kokanee were anesthetized in a 1:20,000 solution of MS222. After the fifth year some of the fish developed fungus infection, which was kept under control by means of a malachite green bath given twice a week.

Survival.—One hundred sixty eight kokanee, 125 males and 43 females, survived operation. Figure 1 shows the typical appearance of the fish at the time of

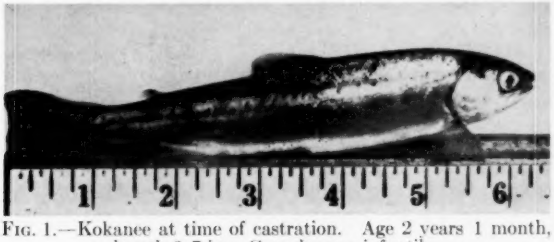

FIG. 1.—Kokanee at time of castration. Age 2 years 1 month, length 6–7 in. Gonads were infantile.

operation.[28] By the fall of 1954 some of the control males, now well along in their third year but still only 7–8 in. long, became sexually mature and died as did one of the castrated males in which a small piece of regenerated testicular tissue was found at autopsy. During the winter of 1954–55 observed deaths were few and almost entirely among the control population. However, a large loss from predation (principally raccoons) occurred. By June, 1955, the census showed that 13 castrated males, 11 castrated females, and 135 unoperated controls had been lost.[11]

TABLE 1

NUMBERS OF SURVIVING KOKANEE AT SUCCESSIVE YEARS

Year of life	Date of population count	Age		Number of castrated males	Number of castrated females	Unoperated controls	Other data
		Yrs.	Mos.				
3rd	5/13/54	2	1	125	43	305	Start of experiment
	10/22/54	2	6	122	37	302	Including loss from predation
4th	6/ 7/55	3	2	108	30	161	
	10/24/55	3	6	103	29	141	Including loss from predation
5th	5/23/56	4	1	22	21	25	
	11/ 4/56	4	7	11	18	18	No predation
	3/ 3/57	4	11	6	15	0*	
6th	5/15/57	5	1	3	11	–	
	10/28/57	5	6	2	8	–	
7th	8/13/58	6	4	2	7	–	
	2/24/59	6	10	2	5	–	
8th	5/20/59	7	1	2	5	–	
	10/21/59	7	6	1	5	–	
9th	4/14/60	8	0	1	1	–	
	7/27/60	8	3	0	1	–	
	10/21/60	8	6	0	0†	–	

* Last control died on this date.
† Last castrated fish died on this date.

TABLE 2

DEATHS AND LOSSES OF KOKANEE AT SUCCESSIVE YEARS

Year of life	Observed Deaths: Castrated: With regeneration of gonads ♂	Castrated: With regeneration of gonads ♀	Castrated: No regeneration of gonads ♂	Castrated: No regeneration of gonads ♀	Unoperated controls with sexual maturation ♂	Unoperated controls with sexual maturation ♀	Predation and other unobserved deaths during winter: Castrated ♂	Castrated ♀	Controls
3rd	1 (K)*	0	1 (K)	1 (K)	6 (2K)	3	15	12	135
4th	46 (2K)	0	2 (1K)	2	27 (3K)	37	38	7	69
5th	9	1	7	5	8 (2K)	18 (1K)	0	0	3
6th	0	8	1	2	0	0	0	0	0
7th	0	0	0	2	...	...	0	0	...
8th	0	0	1 (K)	4	...	...	0	0	...
9th	0	1	1	0	...	...	0	0	...

* (K) = killed for tissue specimens.

These data are presented in Tables 1 and 2. Up to the beginning of the fourth year the castrated fish were indistinguishable in morphology and rate of growth from the controls.

In the fall of 1955 many control kokanee were maturing and dying. They were now in the latter part of their fourth year, when most kokanee spawn in the wild state. Likewise a number of castrated males were showing signs of sexual maturity: prolonged snout, hooked jaws, humped back and eroded skin (Fig. 2). The mor-

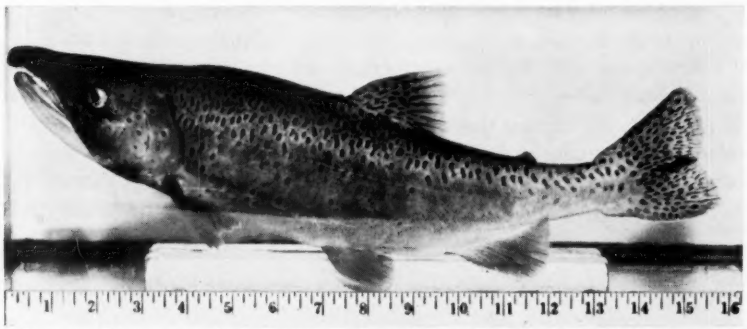

FIG. 2.—Castrated male kokanee showing characteristic morphological changes of normal spawning male—elongated snout, hooked jaws, and hump back. Small fragments of regenerated testes present. Age 4 years 7 months, length 16 inches, at death.

phological changes in these fish were exactly like those occurring in the sexually mature control males. When the castrated male kokanee were examined after death, all but two (Table 2) were found to have regenerated testicular tissue, frequently at the anterior pole of testis attachment but more often in the caudal region adjoining the spermatic duct. Obviously gonadectomy had been incomplete in these fish. In many instances the mass of regenerated testicular tissue was very small, 200 mg or less, whereas the normal mature testes of the control kokanee weighed 9 grams or more. Two castrated females died during their fourth year without regeneration of ovaries. No apparent cause for death was found in these females or in the two completely castrated males which died.

During the winter of 1955–56 the loss from predation was again high (Table 2) despite all preventive measures. By the beginning of their fifth year of life the kokanee population had been reduced principally as the result of sexual maturation plus predation to 22 castrated males, 21 castrated females, and 25 controls. At this time three of the control fish were killed for examination. One was a female with very small eggs, one was an immature male, and the third had no gonads. The number of experimental fish was now sufficiently small so that it was possible to place them in a large outdoor wooden trough 15 × 4 × 4 ft. completely screened to prevent any further predation. The water flow that had supplied the pond was now diverted into the trough.

In November, 1956, it was necessary to transfer the kokanee to another location since the Tahoe hatchery was being closed. The remaining population of 47 fish (see Table 1, Nov. 4, 1956) were taken by tank truck to Crystal Lake hatchery just north of Mt. Lassen some 350 miles distant. The transfer was accomplished without loss and the experimental kokanee were placed in a large screened trough of the same size to which they had been accustomed, and supplied with water at essentially the same temperatures as those at Lake Tahoe. The water flow was 50–60 gallons a minute and the water depth was kept at 26 in.

During the fall and winter of their fifth year all the remaining control kokanee died—the last one, a maturing bisexual fish, succumbed on March 3, 1957, at the age of 4 years 11 months. Autopsies on 143 of the controls showed that the ratio of males to females was 1:3.5, just the reverse of the sex ratio of the castrated population. Twenty-one castrated fish died during this period; all but 2 of the males showed regeneration of testicular tissue, but 5 of 6 females were found to be completely castrated. This left 6 male and 15 female castrated survivors, all of which had a juvenile appearance.

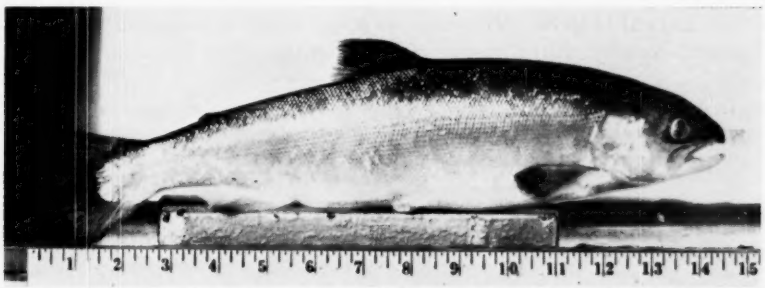

FIG. 3.—Castrated male 5 years and 6 months of age and 14½ inches in length. Silvery color and juvenile appearance.

During the sixth year 10 castrated females died, 8 with regeneration of ovarian tissue (Table 2). In most of them very few eggs were present. One had approximately 400 eggs.[12] Following the death of this female only one of the remaining fish revealed any regeneration of gonadal tissue. Figure 3 shows a castrated male 5½ years old still exhibiting juvenile morphology and a silvery color.

In the seventh year of their lives 2 of the females and 1 male began to develop changes in the shape of the head. One female (Fig. 4) showed a blunting of the nose, and the other, a slight hooking of the jaw, as did the male. The female of

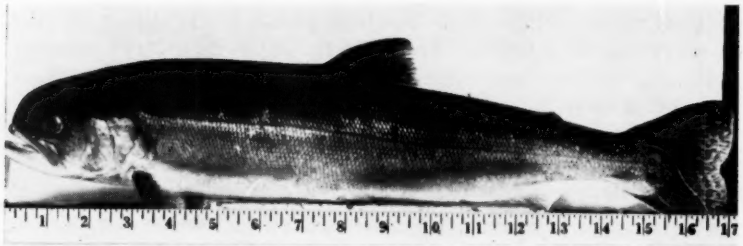

FIG. 4.—Castrated female. Age 6 years 4 months, length 17 inches, weight 880 grams Shows blunting of nose, projection of lower jaw, and loss of silvery color. Died 4 months later, growth to 17 3/4 inches. No regeneration of ovaries.

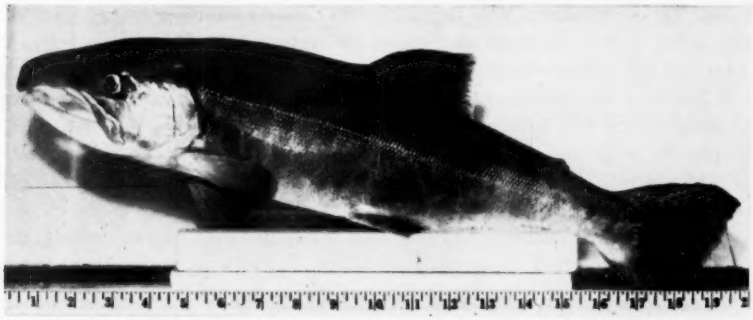

FIG. 5.—Castrated male. Age 6 years 10 months, length 19 inches, weight 1,195 grams. Has appearance of spawning male salmon Sacrificed at 7 years of age as fish was losing weight. No regeneration of testes.

Figure 4 died 4 months after the date of photo, at the age of 6 years 8 months. Hooking of the jaws of the male continued to increase and by the end of the seventh year was marked (Fig. 5). This fish was losing weight rapidly and was sacrificed at 7 years 1 month. Not a vestige of testicular tissue was found at autopsy.

Even in their eighth year several of the kokanee retained their silvery color and juvenile form (Fig. 6). The others began to show some elongation of the jaws,

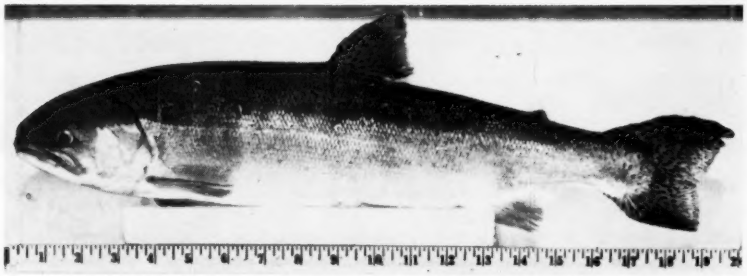

FIG. 6.—Castrated female. Age 7 years, length 19 1/2 inches, weight 1,420 grams. Silvery color and juvenile appearance.

blunting of the nose and darkening of the skin. Four of the 5 remaining females died during this year.

At the beginning of the ninth year only 1 male and 1 female survived. The male was still quite silvery with only a little blunting of the nose but no elongation

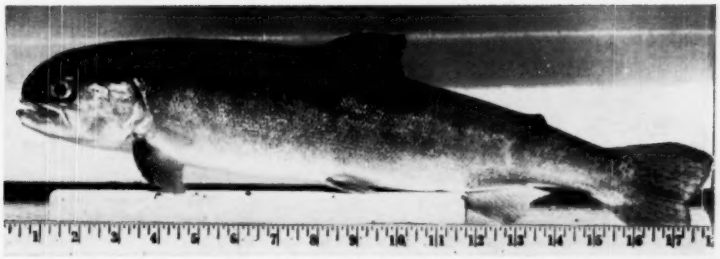

FIG. 7.—Castrated male. Age 8 years, length 17½ inches, weight 845 grams. Still moderately silvery in color. Blunting of nose. Died at 8 years and 2 months, length 18 inches. No testicular tissue found at autopsy.

or hooking of the jaws (Fig. 7). This fish died at 8 years and 2 months of age. No testicular tissue was found. The female was of a grayish color, had well marked prolongation and hooking of the jaws as shown in Figure 8 taken at 8 years 4

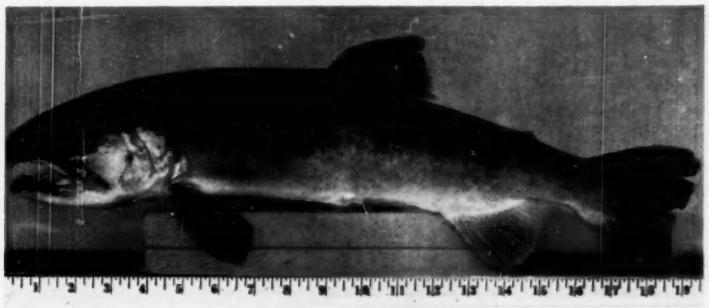

FIG. 8.—Castrated female. Age 8 years 4 months, length 19¾ inches, weight 1,200 grams. Grayish color, elongated, and hooked jaws. Died at 8 years and 6 months. Two small clusters of immature eggs found at autopsy.

months. This fish had been losing weight for the past few months, had sunken eyes and died at 8 years and 6 months of age. A small cluster of regenerated eggs 3 mm. in diameter was present at each upper pole of ovary attachment: 6 eggs in the left and 22 in the right.

Growth.—Increase in length of the experimental fish is shown in Figure 9. The castrated fish, males in particular, grew more rapidly than did the controls. The last 22 control fish at death averaged 11.7 in. in length, whereas 4 castrated males dying at this time averaged over 14 in. Among more than 100 sexually mature unoperated controls, only one reached the length of 14 in. After the 6th year of life the castrated females were slightly longer than the males and continued to grow more rapidly. Both sexes appeared to reach a maximum length in the eighth year of age. Two fish showed continuous increase in length up to the time of death. One, dying at 6 years 8 months, grew ¾ in., even though during the last 4 months it lost 75 grams in weight. The other, which died at 8 years 1 month, grew ½ in. during the last 6 weeks of life while losing 45 grams. The remaining 5 kokanee living beyond 6 years of age showed cessation of growth several months before death.

The castrated fish of both sexes gained more weight than did the controls (Fig.

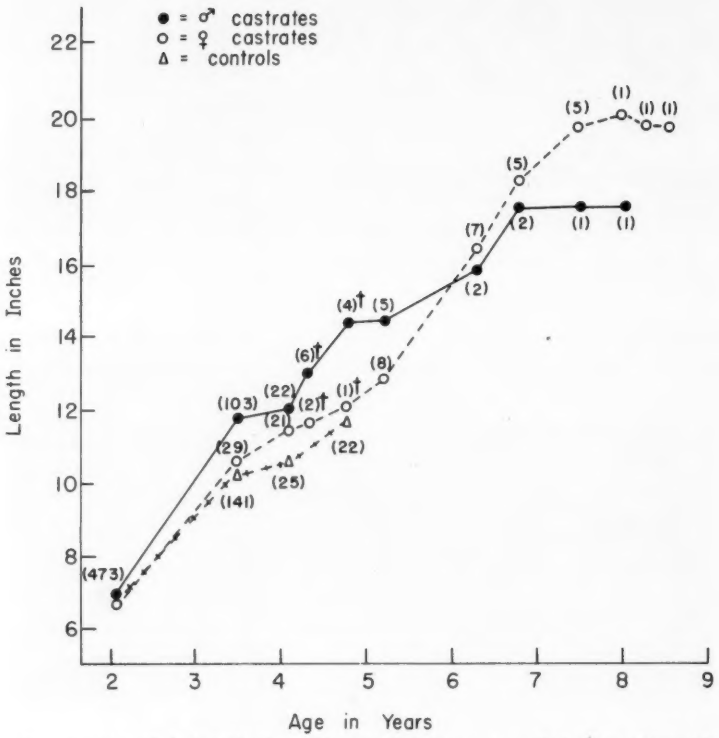

FIG. 9.—Growth in length of experimental kokanee. † = died on or about this date. Others died at irregular intervals.

10). As one would anticipate from their longer length, the males weighed more than the females until the fifth year of life. Beyond this age the females were heavier than the males. However, the number of males, 2, was too small to make a fair comparison. The largest fish, a female, weighed 1650 grams (approximately $3^1/_2$ lb) at the age of $7^1/_2$ years and was $20^3/_4$ in. long.

Other Changes.—Gonads: The mass of ovarian tissue found in those fish showing gonad regeneration varied from as little as 9 mg to as much as 1120 mg and the number of eggs from 5 or 6 to several score. The size of the eggs were with a single exception much smaller than the eggs of the mature control kokanee. Eggs of 29 control fish averaged 4.3 mm in diameter, whereas in 9 castrated kokanee the eggs were from 1–2 mm in 6 and 2–3 mm in 3 of them. One castrated female dying at 5 years 9 months was found to contain approximately 400 eggs measuring 5.5 mm in diameter (the same size as eggs taken from ripe kokanee in the wild state). This was the only castrated female in which the eggs of the regenerated ovary developed to a large normal size.

In contrast to the usual immature state of the eggs of regenerated ovarian tissue, the regenerated testes exhibited full sexual maturity, i.e. spermatozoa, even though the mass of the gonad tissue was very small.

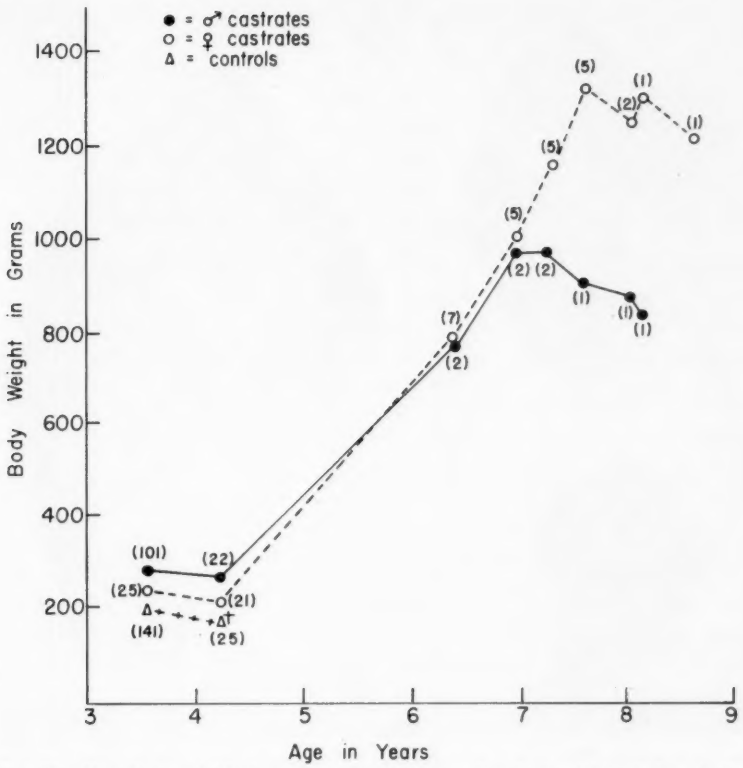

FIG. 10.—Growth in weight of experimental kokanee. † = last weighing before death of all controls in fall and winter of fifth year.

Scales: All the mature control kokanee showed absorption of the scales[13] with one exception, that of a young female dying in the fall of the third year of life. Likewise, all but one of the castrated males showing regeneration of testicular tissue exhibited absorption of the scales equal in degree to that found in mature control kokanee (Fig. 12). On the other hand, scale absorption occurred in only one of the females with regenerated ovarian tissue; this was the fish referred to above which contained a large number of eggs. None of the castrated kokanee of either sex, dying without having regenerated gonadal tissue, exhibited absorption of their scales.

The scales of the very old fish, those dying after the sixth year, showed distortion in shape with perhaps some absorption of the caudal end of the scale, but no obvious irregular loss of circuli such as occurs in the scales of sexually mature kokanee (Figs. 11–13). The scales of the old fish (Fig. 13) were much larger than those of the younger kokanee (Fig. 12). This finding was to be expected since the scale grows pari passu with the total body growth.

Lack of spawning: No evidence of spawning activity was observed among the mature control kokanee. At autopsy the females were found to be full of eggs, either loose in the body cavity or still retained in the ovary. Whether any eggs

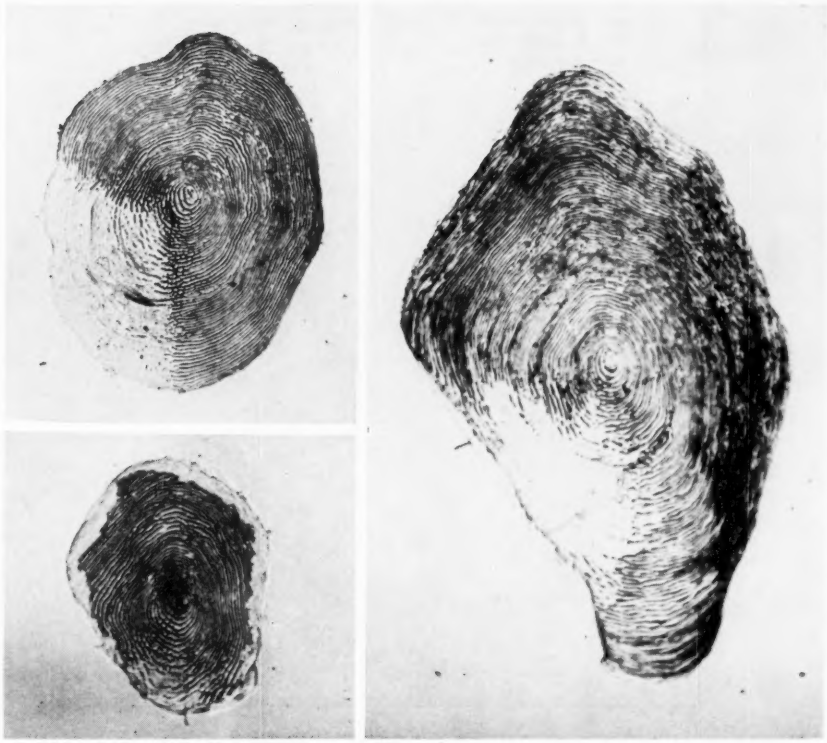

FIG. 11 (*Top left*).—Normal scale of castrated female kokanee which died at 4 years and 2 months of age without regeneration of ovaries. Length, 12 inches. ×16.

FIG. 12 (*Bottom left*).—Scale of male castrated kokanee which died at 3 years and 7 months of age with regeneration of testes. Length $13^1/_2$ inches. Shows loss of peripheral circuli and loss of scale substance. ×16.

FIG. 13 (*Right*).—Scale of castrated female sacrificed at 7 years of age. No regeneration of ovaries. Length $20^3/_4$ inches. Shows marked increase in size over scale of 4-year-old fish and distortion of shape. No absorption except possibly at left lower aspect. ×16.

were shed was not known. The ripe males exuded milt on handling, but how much was shed before death was unknown; the presence of large testes suggested that most of the milt was retained until death. The eyes of the old fish showed no evidence of cataract formation. However, in some of them the eyes were markedly sunken in their sockets.

Histological Changes.—The histological changes occurring in spawning Pacific salmons, including the kokanee, have been described in previous communications.[2, 3] The most striking and significant change is a marked hyperplasia of the adrenal cortical tissue. Castrated kokanee in which regeneration of gonadal tissue occurred showed hyperplasia of the adrenal tissue. In contrast, the completely castrated fish, dying or killed up to the time the last of the control kokanee maturated and died, showed no detectable change in this tissue.[14] A detailed account of the histological alterations which took place in the senescent kokanee will be given in a subsequent paper.[15]

Discussion.—The life span of kokanee under natural conditions appears to depend to a considerable extent on latitude. Curtis[16] found that kokanee in California matured at the end of their third year. His observations included Donner Lake which is near Lake Tahoe and at approximately the same altitude. In the Columbia and Fraser River systems kokanee have been observed to spawn in their third fourth, and fifth years.[17] Mosher, quoted by Galligan,[18] found among approximately 1,000 land-locked kokanee and sockeye salmon taken from lakes of the Northwest only a few 6-year-old and one 7-year-old fish. Farther north in Alaska, sea-run sockeye return to spawn at 3 to 8 years.[19]

That land-locked sockeye salmon in the wild state can survive well beyond their normal life cycle, provided they do not mature sexually, has been reported recently by Galligan.[18] He secured from a lake in Connecticut a large immature female sockeye 20 in. long and weighing 3 lb whose scales showed 8 annuli and no spawning mark. Mature adults of the sockeye population of this lake spawn in the fall of their fourth year and reach a length of 14–16.5 in.[20] The latitude and climatic conditions of the Lake Tahoe and Crystal Lake regions are not appreciably different from those in Connecticut.

Castration of mammals appears to have no significant effect on length of life.[21] However, Korenchevski has observed various indications of accelerated senescence in castrated rats.[22] Likewise, eunuchs show premature aging,[23, 24] but there is no definite evidence that the life span of either rats or man is shortened by gonadectomy. That the effects of castration of kokanee would be other than those resulting from ablation of the gonads is mammals might be anticipated from the difference between Pacific salmon and mammals with respect to the consequences of reproduction. While only a very limited number of fishes die regularly after their first spawning[25] many species show a greatly increased incidence of mortality at this time.[26] It is possible that castration of such fishes at an early age might have the same effect as it did on kokanee.

Our studies on the nature of the post-spawning death of Pacific salmon suggest that hyperadrenocorticism is the principal cause of the fatal outcome,[4] but what precipitates this state remains unknown. One finding in the present investigation provides a possible clue. The principal apparent stress common to both migratory and nonmigratory salmon, namely the formation of a large gonadal mass under conditions of starvation, might seem to be of sufficient intensity to produce the adrenal hyperactivity characteristic of spawning salmon. Yet the castrated male kokanee with regeneration of only a very small amount of gonadal tissue shows marked hyperplasia of the adrenal cortical tissue and dies with all the typical stigmata of the fish with normally mature testes. It is accordingly clear that the stress of gonad formation is not operating in these fish. Is the production and release of gonadotropins by the pituitary accompanied by increased ACTH production, or does some product of testicular growth stimulate the pituitary to greater ACTH secretion or perhaps act directly on the adrenal cortical cells? The possibility that hyperactivity of the adrenal cortex may be incited by factors other than ACTH is indicated by the recent finding of Hilton and co-workers.[27] They showed by means of direct arterial perfusion of the adrenal glands of the hypophysectomized dog that secretion of significantly increased quantities of hydrocortisone could be produced by synthetic lysine, argenine, and acetyl argenine

vasopressin.

The relationship of regeneration of gonadal tissue to death was not apparent in the castrated female kokanee. Up to and including the fifth year of life of the experimental fish, when all the controls had died, only 2 of 9 castrated females showed regeneration of ovarian tissue at the time of death while 56 of 66 dead castrated males exhibited varying degrees of testis regeneration.

Some evidence was provided by an earlier small-scale, and heretofore unpublished experiment, that castration of kokanee with well developed gonads has relatively little effect on lengthening their life span. These fish were gonadectomized when $2^1/_2$ years of age, in the fall of the year. The control males began dying within a month or so after the beginning of the experiment and showed fully mature testes. The majority of the castrated males survived somewhat longer than did the male controls and died without regeneration of testicular tissue. Two of them outlived the last of the male controls by 3 months. However, no difference in length of life between female castrates and female controls was observed and the oldest survivor of the experiment was a control female.

Summary.—To ascertain whether or not there is a causal relationship between the spawning act and subsequent death of Pacific salmon, a large number of kokanee salmon (168) were castrated at 2 years of age while still completely immature. They were maintained in a rearing pond together with twice the number of unoperated controls. The castrates grew well and were indistinguishable from the controls until the fourth year when many of the males took on the morphological appearance of normally maturing salmon, exhibited degenerative changes and died. Regeneration of testicular tissue, usually very small in amount, was found to be present in these fish. The control kokanee all matured and died before the end of their fifth year. A small group of castrates (21) were alive at this time, and continued to grow. Seven of them reached the eighth year of life and two survived until the ninth year. The last, a female, died at 8 years 6 months. The results of this experiment indicate that it is the maturation of gonadal tissue and not the actual spawning (at least in males) which leads to degeneration and death and that if sexual development is prevented by castration kokanee can survive well beyond the normal life span of this species.

* This work was aided by grants from the American Philosophical Society and the National Science Foundation.

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[8] Robertson, O. H., *Fisheries Bull. 127, U. S. Fish and Wildlife Service*, **58**, 9 (1958).

[9] I wish to thank the Upjohn Company of Kalamazoo, Michigan, for the penicillin which was provided through the kindness of Dr. Jared H. Ford.

[10] The author is deeply indebted to many members of the California Department of Fish and Game for their long-continued, generous assistance in carrying out this study. First to Mr. Alan Taft, then chief of the branch of Inland Fisheries, who in 1950 volunteered to rear and maintain kokanee for the experiment at the Lake Tahoe hatchery and had a special holding pond

constructed for this purpose; to Dr. Alex J. Calhoun, who succeeded Mr. Taft, and continued to support the work with all the facilities needed; and to Mr. Donald Evins, and his assistant Mr. Phillip Murray at the Tahoe hatchery, who deserve great credit for successfully rearing and maintaining the experimental kokanee under unusually difficult conditions. After transfer of the kokanee to Crystal Lake hatchery, made possible by the whole-hearted cooperation of Mr. Harry A. Hanson and Mr. Harold H. Hewitt, my thanks are due to Mr. Jack Bell and Mr. D. A. West who succeeded Bell. It was undoubtedly due to Mr. West's constant supervision and skillful care that the aging kokanee were kept in such good condition during their final years. Other members of the Department who gave valuable assistance were Mr. Leo Shapovalov, Mr. Jack Fraser, Mr. Allen F. Pollitt, and Mr. Harold Wolf.

[11] The relatively greater loss among the controls might have been due to the fact that many of them had matured and in this state were sluggish and hence more easily caught by predators.

[12] This is less than half the number of eggs produced by the wild kokanee of Donner Lake.

[13] Scale absorption as designated by fish biologists consists of varying degrees of loss of peripheral scale substance.

[14] The author is indebted to Dr. Bernard C. Wexler, May Institute for Medical Research, Cincinnati, Ohio, for providing a number of these histological preparations.

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[28] I wish to record my gratitude to the late Dr. Clifford Sweet of Oakland, California, who performed many of the castrations.

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